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# Immobilized Enzyme Removal of Colour from Kraft Pulp Mill Effluents in Northern Alberta

by

Joel Edmund Grant

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science in Environmental Science

Department of Civil and Environmental Engineering

Edmonton, Alberta

Fall 2000

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## Abstract

The enzyme horseradish peroxidase (HRP) has been shown to be effective in removing colour from pulp mill effluent. It has also been shown that HRP's effectiveness and active life are increased when it is immobilized on a solid support. In this investigation, five ion exchange resins were tested for their capacity to retain active HRP. An assay was developed to reliably measure the amount of active enzyme retained and it was found that the strongly basic anion exchange resin Amberlite IRA-400 performed best for retaining active enzyme and withstanding fluid shear. The immobilized enzyme was tested for its capacity to remove colour from kraft effluents. It was found that the enzyme was not effective in removing colour, but the ion exchange resin was very effective. These findings provide information on the structure of colour forming bodies in kraft mill effluent; that they are likely not phenolic as was previously thought.

"Enzymes are things invented by biologists that explain things which otherwise require harder thinking."

-Jerome Lettvin

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled *Immobilized Enzyme Removal of Colour from Kraft Pulp Mill Effluents in Northern Alberta* submitted by Joel Edmund Grant in partial fulfillment of the requirements for the degree of Master of Science in Environmental Science.

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*This thesis is dedicated to my parents, Judy and Terry Grant, whose love, support and encouragement have guided me through my whole life.*

## Acknowledgements

The author would like to thank Dr. Ian Buchanan for his excellent guidance throughout this project. Also, the author would like to thank Nick Chernuka and Maria Demeter at the Department of Civil and Environmental Engineering for their technical help and advice.

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# 1 Introduction

The northern regions of Alberta are covered by the boreal forest. This abundance of softwood trees has supported the forest industry for years. There are many sawmills, planermills, oriented strandboard plants and pulp mills scattered across Alberta's boreal forest. The effect on the environment of pulp mills is a particular concern, specifically the effect of effluent wastewater which is released into natural watercourses. The majority of research in this area has been focused on reducing biochemical oxygen demand (BOD) and toxicity of these effluents. Specific contaminants such as 2,3,7,8-tetrachlorodibenzodioxin and dibenzofurans are closely monitored. Another aspect of research in pulp mill effluent discharge is colour. Colour has been the subject of many investigations, as it is a widespread problem in the pulp industry. Whereas colour may not be directly toxic to aquatic organisms, it can be detrimental in that it may reduce light transmission in water. Moreover, colour often has a displeasing aesthetic effect on natural areas. There are proven technologies in use to reduce colour in pulp mill effluents. These technologies include oxygen delignification, membrane filtration, and peroxide addition. The problem with these technologies is that they are most often very expensive to startup and maintain. The pulp industry is very interested in finding other lower cost options to remove colour, as many plants are not equipped with colour removal technologies.

### *1.1 Pulp Mill Effluent*

The effluent of kraft pulp mills is a combination of several process wastewaters. These processes include digesting, brownstock washing, bleaching and drying of pulp. The effluent contains various components such as lignin derivatives and spent pulping liquors. This wastewater has a high organic load and must be treated prior to discharge into natural receiving waters. Concern has arisen over the many potential environmental effects including: abnormal pH, high suspended solids concentration, oxygen demand, toxicity, bioaccumulable organics, mutagenics, and colour (Walden *et al.*, 1985). Treatment of pulp mill effluent is usually accomplished by first removing solids by settling and then applying biotreatment. Biotreatment of effluent prior to discharge greatly reduces the concentration of most of the component organic chemicals, which reduces their potential environmental impacts. However, biotreatment does not remove colour. The organic molecules which cause colour are too large and bulky to be broken down by the microorganisms in a pulp mill waste treatment system in the relatively short contact time available.

### *1.2 Enzymatic Treatment*

Since the 1970's, researchers have been investigating enzyme-catalyzed treatment processes specifically designed for selective removal of compounds found in industrial wastewaters (Aitken 1993). Pure enzymes isolated from their parent organisms are often commercially available and offer several advantages over

biological treatment. Some of these advantages are that isolated enzymes (Nicell *et al.*, 1993b):

- May remove a broad range of contaminants;
- May be applied to remove a single target compound;
- Can respond quickly to shock loadings;
- Require no acclimation time; and
- Are effective over broad ranges of pH and temperature.

Enzymatic processes can also have advantages over physical and chemical processes as well. Some of these advantages are that enzymatic treatment (Nicell *et al.*, 1993):

- Is able to remove organics which are not readily oxidized;
- May be highly selective;
- Can be effective at high and low substrate concentrations; and
- Can operate with less oxidant than conventional chemical oxidation methods.

One group of enzymes shown to be effective in removing aromatic compounds from wastewaters is oxido-reductive enzymes, specifically the peroxidases. These enzymes utilize hydrogen peroxide to oxidize aromatic compounds. The aromatics are converted into free radicals which spontaneously form polymers. These polymers grow to larger and larger lengths until their solubility is reduced to the point where they precipitate out of solution (Nicell *et al.* 1995). One such enzyme which has been shown to be effective in treating pulp mill wastewaters is Horseradish Peroxidase (HRP). HRP has been used to remove phenols and chlorophenols (Davis and Burns 1990, Nakamoto and Machida 1992) as well as

colour from pulp mill effluent (Ferrer *et al.* 1991). HRP processes have been optimized and enzyme inactivation reduced by the use of protective additives such as polyethylene glycol (Nakamoto and Machida 1992). While these applications are encouraging, further research is required to assess the applicability of this technology to the kraft pulp industry in Alberta.

### *1.3 Research Requirements*

Research has indicated that HRP can be an effective means of removing colour from pulp mill effluent (Ferrer *et al.* 1991; Peralta Zamora *et al.* 1998). However, effluent components can vary greatly from mill to mill. Lignin, the material which holds wood fibers together which is broken apart in the pulping process, varies greatly with climate, wood type and the geology of the area (Walden *et al.* 1985). Thus, it can not be predicted that because HRP removed colour from effluent in a kraft pulp mill in Brazil (Peralta Zamora *et al.* 1998) or in Chile (Ferrer *et al.* 1991) that it would definitely be effective on kraft pulp mill effluent in northern Alberta.

In designing a reactor, a soluble enzyme would be difficult to keep inside the reactor. It is known that immobilizing an enzyme (attaching it to a solid support medium) can extend the life of the enzyme (Peralta Zamora *et al.* 1998). Peralta-Zamora *et al.* (1998) quite effectively immobilized HRP on ion exchange resins, and used the enzyme to remove kraft pulp mill effluent colour. The resins retained a large amount of active enzyme. In this investigation, various resins will be tested for their potential to retain active HRP. Reliable methods of measuring immobilized enzyme activity are currently lacking. Therefore a new method should be developed.

The ion exchange resins used to immobilize HRP in previous studies are not available in North America. Therefore, research to identify a suitable resin is required.

### *1.4 Scope and Objectives*

The objectives of this investigation were as follows:

- To immobilize HRP on various ion exchange resins;
- To develop a reliable assay to measure the activity of immobilized enzyme;
- To determine which of the exchange resins tested retains the most HRP;
- To evaluate the effect of shear on the immobilized HRP;
- To investigate the ability of the immobilized enzyme to remove colour from kraft mill effluents;
- To determine the optimum point within the mill to apply the treatment; and
- To determine the relative contributions of the HRP, peroxide and resin to colour removal.

After testing a number of resins, the resin which is able to retain the most HRP was used for a series of colour removal trials to determine the optimum conditions for colour removal. By comparing results with blank trials, it was be possible to determine the relative contributions to colour removal of the HRP, hydrogen peroxide (which itself is a strong oxidant known to remove colour) and the ion exchange resin. If the immobilized enzyme shows great potential for colour removal, future study may involve reactor design.

It should also be noted that the methods developed in this investigation were to be mindful of cost. Enzymatic treatment of pulp mill effluent must be designed to be low cost and reliable.

# 2 Literature Review

## *2.1 The Kraft Pulping Process*

The manufacturing of kraft pulp is a multistage process. The object of pulping is to separate the cellulose fibers from the lignin which binds the fibers together. The resulting pulp is used in papermaking and the manufacture of other products such as disposable diapers, and paper toweling. Kraft pulp is specifically manufactured for its high tensile strength, brightness and capacity to absorb water (Walden *et al.* 1985). Kraft pulp is most often manufactured using an alkaline chemical process. The two Alberta bleached kraft pulp mills from which samples were taken in this investigation use this alkaline chemical process to produce bleached kraft softwood pulp.

The first stages of pulp production involve preparation of the raw material. Trees are cut, delimbed and debarked. The logs are then cut into small "chips" before being fed into a large digesting chamber. In the digester, the chips are subjected to high pressure and a harsh chemical solution called white liquor consisting of sodium hydroxide, sodium sulfate and other alkaline chemicals (Walden *et al.* 1985). This process turns the chips into a temporary phase called brownstock. The brownstock is washed and then moved to a bleaching stage.

Bleaching can be accomplished via a variety of steps and chemicals, most commonly chlorine and chlorinated compounds. In most cases acidic bleaching steps of elemental chlorine or chlorine dioxide are sequenced with alkaline extraction steps. These steps produce the majority of the colour load of the final mill

effluent (Ferrer *et al.* 1991). In the past, elemental chlorine was used in kraft mills in Alberta. However, environmental studies found that the use of elemental chlorine contributed to detectable levels of dioxins and furans, which are very toxic, in the receiving waters. Specifically of concern was the chemical 2,3,7,8-tetrachlorodibenzodioxin, which disrupts neurological pathways in humans and animals by inhibiting cytochrome P-450 (Bunce 1994). These dioxins form spontaneously when parent compounds such as polychlorinated biphenyls are present. In order to prevent these toxic chemicals from forming, Alberta kraft mills switched by 1992 to total elemental chlorine free (ECF) bleaching, where chlorine dioxide is used in the bleaching steps rather than chlorine. Levels of dioxins soon dropped below detection limits in the receiving waters of the mills.

After bleaching, the pulp is moved along sheets and dewatered. As the pulp is dried, it is formed into sheets. These sheets are moved through a drying machine and finally cut into shippable sizes and stacked and transported to the user.

The spent liquor from the digesting stage comes out black. This black liquor is then recycled in what is called the liquor loop. The black liquor is burned in the mill's recovery boiler, which produces energy. After burning and condensing, the liquor is green. The green liquor is recaustisized by adding lime. After the addition of lime, the liquor is ready for reuse as white liquor (Walden *et al.* 1985).

From the pulping process described above, there are several process wastewater streams which combine to make pulp mill effluent. The main contributors to pulp mill effluent are brownstock washings, bleach plant effluents and water removed during the drying stage. The majority of the colour is from the

bleaching effluents. Pulp mill effluent contains many different contaminants which cause environmental concern. As mentioned in Section 1.1, major concerns include high BOD loading due to the organic content, suspended solids, toxicity and mutagenicity. The number of contaminants is very large and is variable, including many resin acids, quinones and ketones. (Walden *et al.* 1985) Biotreatment of the effluent is effective in removing most of the BOD load and toxicity of the effluent by breaking down the molecules, but does not act on colour.

### *2.2 Pulp Mill Effluent Colour*

Effluents from pulp mills possess a dark brown colouration. This colour comes from different stages of the pulping process, the majority from the bleaching steps. Pulp mill effluent colour may inhibit the transmission of light in natural waters, thus causing potential damage to aquatic plants and other organisms. Also, colour causes an aesthetic problem in natural areas. The colour itself is attributed to solubilized lignin residues from the pulping and bleaching processes (Walden *et al.* 1985). The specific compounds which form the colour are not known. They have been described as chromophoric lignin (Royer *et al.* 1990). It is believed that the residues which cause colour are comprised of coniferaldehyde units, aryl ketones, quinone methides, and quinones (Walden *et al.* 1985). These quinones are derived from the original lignin, as well as from the degradation of cellulose and sugars, the auto-oxidation of catechols, and the demethylation of guaiacyl and syringyl groups in the lignin. The structure of the lignin and its residues will vary with the type of wood being pulped (Walden *et al.* 1985). Colour bodies can be divided into two groups:

(i) low molecular weight compounds, which have fewer aromatic rings, and (ii) high molecular weight compounds, which make up the bulk of the colour (Walden *et al.* 1985). It is also believed that phenols and chlorophenols are major building blocks in pulp mill effluent colour. Ferrer and coworkers (1991) wrote that phenolic residues released in the alkaline extraction stages of wood pulp bleaching account for over 50% of the colour load. The task of identifying the precise structures which form colour has not been undertaken. Kemeny and Banerjee (1997) studied the relationships between effluent constituents and COD in kraft mill effluents and found that colour levels correlated well with COD levels. That study suggests that colour originates from carbon based compounds that are not removed during treatment. Kemeny and Banerjee (1997) also report that colour occasionally increases across a waste treatment system (such as a lagoon), and therefore a portion of the organic material is not mineralized but instead converted into more chromophoric units.

Several strategies to remove colour from pulp mill effluent are currently available; physical processes such as membrane filtration, chemical processes such as ozonation, and biochemical processes such as enzymatic removal of contaminants. Despite the availability of options for colour removal such as those named above, colour remains a problem with many kraft mills, including those in Alberta. Membrane filtration is a process known to be very effective in removing colour. This process involves forcing bleach plant effluent through ultrafiltration or reverse osmosis membranes which remove impurities, including the colour forming bodies. Its advantage is high removal of colour; its disadvantages are a very high

startup cost and the need for frequent backwashes to maintain the membranes (Walden 1985).

The chemical process of ozonation is also used in the pulp industry to remove colour. Ozone, a strong oxidant, is bubbled through effluent streams. The ozone breaks down a large number of organics, including colour forming bodies. Advantages of ozonation include good removal of colour and reduction of BOD concurrently. Disadvantages of ozonation are the high startup and operating costs. These costs are enough to drive research in other areas (Walden 1985).

A popular method of colour reduction used at one mill in Alberta is oxygen delignification. This process, like ozonation, is a chemical process. Oxygen is bubbled through the pulp slurry before the bleaching process after the brownstock washing step. Oxygen is an oxidant which helps to break down the lignin molecules into smaller molecules which do not cause colour. This process is quite reliable to remove colour, although it too is a costly technology.

These methods described all carry large startup costs for the companies running the mills. With operating permits becoming more and more restrictive with colour discharge limits, and the extreme costs of the established methods, it is pertinent to research innovative methods to remove colour from kraft mill effluent. One potential method for research is the use of enzymes. In this investigation, the enzyme horseradish peroxidase was tested for applicability for use on kraft mill effluent in Alberta.

### *2.3 Horseradish Peroxidase*

Horseradish peroxidase (HRP) is a peroxide dependent enzyme. Its catalytic site consists of a hemic iron inside a protoporphyrine structure (Peralta Zamora *et al.* 1998). It is a very nonspecific peroxidase, catalyzing a large number of reactions on a variety of substrates, such as phenols and chlorophenols, amines and other aromatic compounds. HRP has been used to remove aromatic amines and phenols from industrial effluents (Davis and Burns 1990). Advantages of using HRP for industrial waste treatment also include (Nicell *et al.* 1992):

- HRP remains active over a wide range of temperature, pH, and salinity;
- HRP is often slow to be inactivated; and
- HRP is widely commercially available.

There are potential disadvantages to the use of HRP in industrial processes. Among these are the high cost of the enzyme itself, the possible formation of solid products which are more toxic than their parent compounds, and the disposal of these solid products. However, additives such as polyethylene glycol (see Section 2.4) have been shown to greatly extend the catalytic life of HRP. Given the wide range of substrates HRP can remove from solution, and the complex makeup of pulp mill effluent, it is pertinent to investigate the possibility of using HRP to remove colour from the kraft mill effluent produced in northern Alberta.

### *2.4 The HRP Catalytic Cycle*

Horseradish peroxidase removes phenolic and other aromatic compounds from aqueous solutions via a polymerization process. In this process, HRP is oxidized by

the strong oxidant hydrogen peroxide ($H_2O_2$) from its ground state, called $HRP_n$, to form a catalytically active compound called Compound I ($HRP_i$). Compound I can be further altered by converting a phenol or phenolic substrate ($AH_2$) into a free radical ($AH^{\cdot}$), converting the enzyme into its Compound II form ($HRP_{ii}$). Compound II also converts a phenolic substrate into a free radical ($AH^{\cdot}$), and in doing so is returned to the enzyme's ground state. This sequence is shown below in Equations (2.1) to (2.3) (Buchanan 1996).

$$HRP_n + H_2O_2 \overset{k_1}{\Diamond} HRP_i + H_2O \qquad (2.1)$$

$$HRP_i + AH_2 \overset{k_2}{\Diamond} HRP_{ii} + AH^{\cdot} \qquad (2.2)$$

$$HRP_{ii} + AH_2 \overset{k_3}{\Diamond} HRP_n + AH^{\cdot} + H_2O \qquad (2.3)$$

Equations (2.1) to (2.3) are the main reactions in the catalytic cycle of HRP. However, there are also side reactions which compete with the main reactions to inactivate or inhibit the useful function of the enzyme. When Compound II is oxidized by hydrogen peroxide it forms Compound III ($HRP_{iii}$), which is inactive (Nakajima and Yamazaki 1987).

$$HRP_{ii} + H_2O_2 \overset{k_{app}}{\Diamond} HRP_{iii} + H_2O \qquad (2.4)$$

While Compound III is inactive, Nakajima and Yamazaki (1987) found that Compound III will spontaneously revert to the ground state ($HRP_n$) according to the reaction:

$$HRP_{III} \xrightarrow{k_{eff}} HRP_n + {}^{\cdot}O_2^{-} \qquad (2.5)$$

Another side reaction can involve Compound I with hydrogen peroxide in the absence of a reductant substrate. What is formed is a complex of Compound I and peroxide, ($HRP_I \bullet H_2O_2$), in an irreversible reaction. This product can subsequently be converted to $HRP_n$.

Alternatively, Compound I can follow a pathway which leads to the production of a permanently inactive form called verdohaemoprotein (P-670) (Arnao *et al.*, 1990). In the presence of a reductant substrate, the amount of $HRP_I \bullet H_2O_2$ is negligible (Nicell *et al.*, 1992). HRP is also inactivated by extremes of pH and temperature, as discussed in Section 2.7.

HRP can also be affected by altering pH. Nicell and co-workers (1993b) studied phenol removal with altering pH from 2 to 10 and found a local optimum around pH 4.0, and a global optimum removal pH around 8. Removal decreases as pH moves away from the optimum. This will be discussed in more detail in Section 2.7.

A diagram of the full catalytic cycle showing all reactions is shown in Figure 2.1:

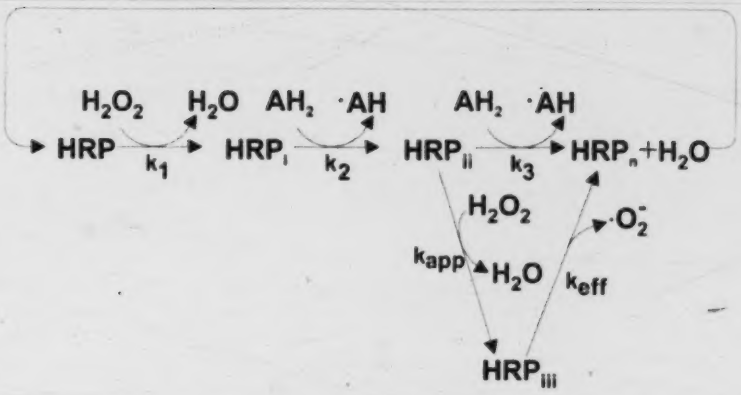


**Figure 2.1 HRP Catalytic Cycle.**

The reactions which most concern this study are equations (2.2) and (2.3) which form free radicals. These free radicals, once freed from the enzyme form stable dimers (Yu *et al.* 1994). These dimers can again react with Compounds I and II to form free radicals and undergo further polymerization. Also, free radicals (also depicted as $AH^{\cdot}$) can also transfer electrons to form polymer radicals, which result in larger polymers. The growth of these polymers can be expressed by the reaction:

$$\text{Free Radical} + \text{Free Radical} \xrightarrow{k_p} \text{Polyaromatic Product} \quad (2.6)$$

As the polyaromatic products become larger and larger, their solubility decreases until they eventually precipitate from solution and can be removed by physical processes (Buchanan 1996).

The inactivation of HRP is greatly accelerated by the presence of a reductant substrate such as the polymers formed from the phenolic substrates and free radicals (Buchanan 1996). The interaction of HRP and free radicals is the major inactivation pathway for HRP. With pulp mill effluents containing many substrates which would form free radicals, there is a strong possibility that the HRP would all be quickly inactivated. Thus, it is important that all measures be taken to lengthen the active life of the enzyme.

### *2.5 Effect of Polyethylene Glycol (PEG)*

It has been shown in previous studies that certain chemical additives can extend the active life of HRP. Specifically, high molecular weight polyethylene glycol (PEG) (3500 g/mol) has been shown to be very effective. Buchanan (1996) modeled HRP kinetics in designing reactors using PEG. Nakamoto and Machida (1992) found PEG to be very effective and hypothesized that the reason HRP had a longer effective lifespan was due to the PEG competing with the enzyme for adsorption sites on the polymerized reaction products. It was reported that only a tiny fraction (1/200) of HRP was required to remove phenol from aqueous solution when PEG was present in the solution at a concentration of 4 g/L and when the phenol concentration ranged from 100 to 1000 mg/L. It was observed in studies by Wu *et al* (1993) and by Nakamoto and Machida (1992) that PEG effectiveness increased with increasing phenol concentration. In the interest of designing a colour removal system which is as cost-effective as possible, it is imperative to investigate the use of PEG for its potential to extend the useful life of the enzyme.

## *2.6 Immobilization Techniques*

### 2.6.1 Overview of Immobilization

Enzyme immobilization is a widely used technique to extend the active useful life of an enzyme. It can be defined as the imprisonment of an enzyme molecule in a distinct phase such that it can interact actively with the bulk phase containing the substrate, co-factors or inhibitor molecules (Trevan 1980). The distinct phase in which the enzyme is immobilized is usually water insoluble (Trevan 1980). Rosevear (1987) lists the following benefits of enzyme immobilization:

- retention of the enzyme in the reactor;
- high concentration of the enzyme in the reactor;
- control of the enzyme microenvironment;
- quantitative and rapid removal of the enzyme; and

Also listed by Rosevear (1987) are limitations involved with immobilized enzymes, which are:

- loss of catalytic activity during immobilization;
- mass transfer problems, such as inadequate contact between enzyme and substrate in a reactor; and,
- empirical nature of immobilization technology.

The purpose of this investigation is, as mentioned earlier, to investigate the use of an immobilized enzyme reactor to treat kraft pulp mill effluent for colour removal. In

order to design a reactor that uses HRP, and can remain cost effective, the enzyme's active life would need to be extended as long as possible. Thus, the benefit of retention of the active enzyme in the reactor is paramount.

### 2.6.1.1 The Main Methods of Immobilization

In general, there are only a few methods of immobilizing enzymes to an insoluble support. Basic classification of immobilized enzymes is shown in Figure 2.2 (Trevan 1980):

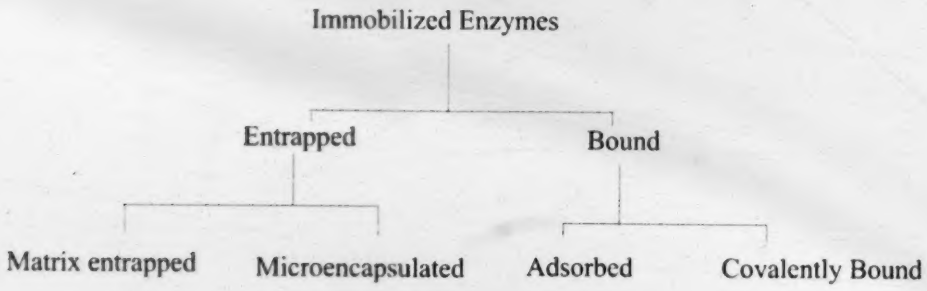


**Figure 2.2 Classification of Immobilized Enzymes.**

#### 2.6.1.1.1 Covalent Bonding to Activated Polymers

The most commonly used method of immobilizing enzymes is through covalent bonding to a carrier polymer (Trevan 1980). Covalent bonding is the sharing of an

electron pair to form a chemical bond which holds the two bodies together. The enzyme bonds itself to the active site and is thus no longer soluble. Cellulose is a polymer matrix which is commonly used. It has been found that the level of activity in the immobilized enzyme depends on the amount of hydration in the polymer matrix. High hydrophilicity enables higher enzyme activity, presumably due to greater enzyme-substrate contact (Trevan 1980).

2.6.1.1.2 Physical Adsorption

This method is the oldest in immobilization and the simplest to perform. In the physical adsorption method, the enzyme is bound to a polymer matrix by a method other than a covalent bond. Binding forces other than covalent can be ionic, hydrogen bonds, van der Waals' interactions and hydrophobic interactions. Immobilization by physical adsorption is attained by simply stirring the enzyme together with the adsorbent (Trevan 1980). However, simple adsorption yields a low amount of enzyme bound per unit of adsorbent and the enzyme may be easily desorbed during reaction in some cases. Cellulose is often used as an enzyme support with this method, in combination with the covalent bonding to immobilize enzyme, but has been shown to be inferior to other supports such as polystyrene resins, collagen, silica gel, alumina and glass.

2.6.1.1.3 Co-Polymerization with Multifunctional Reagents

Enzymes may also be immobilized by linking to multifunctional reagents such as glutaraldehyde (Trevan 1980). In these cases, the enzyme molecules can not only link to polymers such as cellulose, but the enzyme molecules can link to other enzyme molecules. It is possible to create a matrix of only enzyme molecules this way, which can be avoided by using an inert protein for co-polymerization.

2.6.1.1.4 Entrapment and Occlusion

The other means of immobilizing an enzyme are the mechanisms of entrapment and occlusion. There are three possible ways to achieve this (Trevan 1980):

- Inclusion of the enzyme within the matrix of a highly cross linked polymer.
- Separation from the bulk phase by a semipermeable microcapsule.
- Dissolution in a distinct non-aqueous phase.

In the entrapment methods, it should be noted that the enzyme is not actually bound to the support medium, but rather retained in the cross linked matrix. This method is achieved by dissolving enzyme in a solution required for the synthesis of the enzyme phase and treating it so the matrix will form as the distinct insoluble phase. Microcapsules can be formed around droplets of enzyme solution by compounds such as nylon. As these microcapsules are semipermeable, the enzyme can remain active inside. Enzymes can also be occluded in liposomes and micelles, which are spheres of phospholipid bilayers containing aqueous compartments inside hydrophobic exteriors (Trevan 1980).

### 2.6.2 Immobilization of HRP

Previous studies have successfully immobilized HRP. In the following sections various techniques which have been used to immobilize HRP will be discussed.

#### 2.6.2.1 HRP Immobilized by Entrapment

In a study by Davis and Burns (1990), HRP was immobilized by two different methods. The first method was to co-polymerize the HRP with L-tyrosine and subsequently immobilize the complex via entrapment within in 3% (w/v) calcium alginate gel beads cured with 2% $CaCl_2$. The second method was to immobilize the HRP alone in 3% (w/v) calcium alginate gel beads cured with 2% $CaCl_2$. The enzyme was then used for decolourization of phenolic effluents, including pulp mill effluents. The results of decolourization trials with immobilized enzyme were compared with soluble HRP. Soluble HRP removed colour from the effluent, but immobilized enzyme increased the colour removal in the bleach plant effluent by a factor of 20. In the case of the co-polymerized HRP-tyrosine complexes, the increased efficiency factor was 132 over soluble HRP which contained the same amount of active enzyme. The entrapped HRP showed an average removal of 184 colour units after 3 days. The average removal using the entrapped enzyme-tyrosine co-polymers was 1185 colour units over the same time period.

Clearly the method used by Davis and Burns was a potential candidate for study in this project. However, the method for immobilization is complex and involves many steps. Being mindful of the scope and objectives of this investigation it was considered more appropriate to study an immobilization method that could be

performed in fewer steps, at less expense. Therefore, other methods for immobilizing HRP were investigated to find a more suitable candidate.

#### 2.6.2.2 HRP Immobilized by Entrapment and Adsorption

In a study done in Brazil to test the ability of HRP and other peroxidases to remove colour from kraft mill effluent (Ferrer *et al.* 1991) HRP was immobilized on a gel compound called CNBr-Sepharose 4B. Immobilization was accomplished by washing and re-swelling the gel and mixing it in a suspension of buffers with purified enzyme sample. The immobilization process was found to be 38% efficient for HRP, retaining 0.38 active units per gram of dried gel against a theoretical maximum of 1.0 unit per gram of dried gel. In this experiment soluble HRP removed kraft effluent colour to a maximum of 25% of an initial 1894 colour units after 72 hours treatment. In the case of the immobilized HRP, colour removal reached 61% after 72 hours. These results show an excellent potential for the use of immobilized HRP on kraft mill effluent. It is encouraging that the enzyme remained active after a contact time of 72 hours.

As was the case in the previous example, this immobilization method has been shown to work well. However, it also involves a very elaborate procedure for the immobilization process involving many chemicals. If similar results could be attained with fewer steps and less potential costs that method would be more useful than the Sepharose method described above. Moreover, the retained activity achieved here is only 0.38 units per gram. If a different method could be found

which attained a higher retention of activity it would likely be more suited to study in the investigation of the potential of a low cost enzyme technology.

#### 2.6.2.3 HRP Immobilized by Physical Adsorption

A study done in Windsor, Ontario, Canada was performed using immobilized HRP in different reactor configurations and support media for the removal of 4-chlorophenol from aqueous solution (Siddique *et al.* 1992). HRP was immobilized via physical adsorption to three different supports: cellulose disks, nylon balls and nylon tubing. For the nylon supports, their surfaces were subjected to hydrolysis and reaction with glycidol, followed by peroxidate oxidation and reductive amination. For the cellulose disks, periodate-oxidized enzyme sample was coupled to oxidized filter paper treated with a dihydrazide complex.

The support media holding immobilized enzyme were used in three different reactor configurations. For some instances, notably when cellulose disks were used, retained activity of up to 2.66 units was achieved for the system which removed up to 89% of the initial concentration of 4-chlorophenol after a contact time of 6 hours. This study concluded that immobilized HRP showed great potential for removal of chlorophenols and related compounds (such as 2,4 DCP, 2-cresol, etc) found in various industrial effluents, including pulp mill bleach plant effluents. It is believed that chlorophenols are a major contributor to the colour load in pulp mill effluent (Ferrer *et al.* 1991).

The substrate used in Siddique's study is 4-chlorophenol, which is a component of pulp mill bleach plant effluents. However, there is no guarantee that

4-chlorophenol exists in all kraft effluents or that it always a major contributor to colour. Ferrer's article (1991) lists chlorophenols as areas of concern due to their toxicity, and increasing toxicity with increasing chlorine content. If the compounds responsible for forming colour are comprised in part by chlorophenols as is believed, then HRP should indeed have a marked effect on the removal of colour in effluent. The process of immobilization used is again an elaborate procedure, consuming a great deal of time and utilizing many different chemicals in several steps. For the purpose of studying the kraft mill effluent in Alberta, this method of immobilization is unsuitable. The ultimate goal is a simple and effective means of immobilizing the HRP at low cost with high retained activity. A more suitable method is described below in section 2.6.3.4.

#### 2.6.2.4 Immobilization by Ion Exchange (Physical Adsorption)

In a different study in Brazil (Peralta Zamora *et al.* 1998) HRP was immobilized via ion exchange to various resins. After reviewing results of other processes, it was decided that the best methods of immobilization for HRP are the metallic bond, the covalent bond and the ion exchange process. The relevant method used in Peralta Zamora's study is the ion exchange process, performed on the resins IRA-400, IRA-904, CG–400, and CG-120, all Amberlite anion exchange resins commercially available. The method used to immobilize the HRP was straightforward; 1g of each resin support was stirred at 4°C for 20 hours (or 1 hour in the case where the support experienced the unfavourable effect of being broken into smaller and smaller fragments). After stirring, the solid was separated, washed and dried by suction.

This single gram of resin was found to be able to retain up to 100% of the 694 units of activity in the stock solution. These were the best preliminary results of the Peralta Zamora study. IRA-400 was used for a factorial experiment to decolourize kraft effluent varying the amount of enzyme added, the amount of hydrogen peroxide and time of reaction. It had been observed that for textile effluents the decolourization was due primarily to adsorption of the colour forming bodies to the resin itself and not the enzymatic action. However, it was reported that for kraft mill bleach plant effluents and black liquor decolourization occurred with no significant adsorption onto the resin and no effect due to hydrogen peroxide. Decolourization of up to 52.8% was observed for kraft bleach plant first alkaline extraction (E1) effluent after only 160 minutes of contact time coupled with near 80% removal of total phenols. Peralta Zamora's experiment (1998) also studied the effects of a photochemical pretreatment which increased the decolourization results up to near 80% for a three hour treatment.

The results presented by Peralta Zamora's study are largely encouraging. The immobilization process is very straightforward; it is simply stirring the enzyme solution with support medium. Ion exchange resins are widely available commercially at low cost and are already in use in pulp mills for deionization of process water. Colour removal results obtained in this study were also encouraging, removing significant amounts of colour in a short period of time. IRA-400 ion exchange resin appears to be the most promising medium for study of the kraft mill effluents in northern Alberta. The IRA-400 used by Peralta-Zamora was in the hydroxide ($OH^-$) form. As of 1999, this form of IRA-400 had been discontinued and

was not available from Sigma Aldrich Chemicals. However, the chloride ($Cl^-$) form is readily available and is manufactured by the Rohm and Haas company under the name Amberlite IRA-400. Even if the chloride form can only retain a partial amount of the activity retained by the hydroxide form, an investigation into its potential use is clearly warranted. Based on its high capacity to retain active enzyme, its simple process of immobilization, and its potential to remove colour, IRA-400 has been selected to be the immobilization medium further investigated in the current study.

### *2.7 Effect of pH and Temperature on HRP*

Enzymes in general function best at an optimum pH. Nicell and co-workers (1993b) conducted several studies with HRP over a wide range of pH values and found that HRP remains active over the pH range 6 to 9 for significant removal of phenol, as well as several chlorophenols and methylphenols. Of interest to this study, the pH values of kraft pulp mill wastewaters fall in general between 6 and 8. The phenols thought to be colour bodies in the effluents are large bulky ones. It would not be necessary to adjust the pH of the effluent for HRP to function, as it already falls within the active range.

Nicell and co-workers (1993b) also studied the effects of temperature on HRP activity. The activity was observed to increase up to 50°C and then to decrease, as thermal inactivation continues to increase. In other studies by the same group, HRP was found to be active over a range of 5-65°C. Because pulp mill effluent varies in temperature according to the season and proximity to the plant, and this is a preliminary study, the tests will be conducted at room temperature (25°C), which

falls within the active range of HRP. Typical effluent temperatures as it exits the plant falls between 35-60°C, but is reduced during the biotreatment process to near 25°C in the summer, and 15°C in an Alberta winter. The biotreatment process is outdoors and exposed to the atmosphere, which causes the cooling of the effluent. Thus, more cooling occurs in the cold winter months.

### *2.8 Methods of Determining Immobilized HRP Activity*

In order to determine properly the amount of active enzyme retained on a solid support medium, a reliable method is required that provides accurate and repeatable results. The retained activity of HRP after immobilization has been determined in previous studies by a variety of methods.

One method used for determining the amount of enzyme retained was employed by Peralta Zamora *et al.* (1998) involving measuring activity differentials. As described in Section 2.6.3.4, immobilization was achieved by mixing enzyme solution with ion exchange resin. After the time allotted for immobilization had expired, the resin was filtered and the supernatant liquid was collected. This supernatant liquid, along with washings, was assayed for soluble HRP activity. The difference between the stock solution activity and the amount remaining in the supernatant and washings was interpreted as the amount of enzyme retained. This method is simple, using a soluble HRP determination method; however, it does not distinguish between retained active enzyme and retained inactive enzyme.

The method used by Siddique (1992) involved a colour generating assay. This assay forms a chromogen which displays a sharp peak at 510 nm from a

reaction mixture of 2.4 mM 4-aminoantipyrine, 9.0 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid, and 0.1 M phosphate buffer of 7.5. This method was used for three different support media; cellulose disks, nylon balls and nylon tubing. For the cellulose disks, a continuous flow reactor system was employed and the effluent measured for the presence of the chromogen by absorbance at 510 nm. For nylon balls a batch reactor system was employed and the enzyme activity in the supernatant liquid was compared with initial conditions. Activity estimation of the nylon tubing was determined by a batch reactor system with recycle. Part of the colour generating assay solution was routed through a flow cell in a spectrophotometer where absorbance was measured at regular intervals.

The cellulose disk method is the only one described which does not use a differential method similar to that of Peralta Zamora (1998). That is, it is the only method which does not contain the potential error of retained inactive enzyme. Another disadvantage of all these methods is that no attempt is made to determine the amount of enzyme sheared off while attempting to determine retained activity. Any flowing liquid will exert a shearing force on attached enzymes. The methods used by Siddique (1992) and Peralta Zamora (1998) do not make any attempt to determine if the shearing forces remove enzyme from the immobilization media.

# 3 Materials and Methods

In this investigation, established methods were used to quantify the activity of soluble enzyme and to determine colour levels. For determination of immobilized enzyme activity, a new method was developed and is described below.

## *3.1 Materials*

Horseradish peroxidase enzyme (HRP), catalase enzyme, Amberlite ion exchange resins IRA-400, IRA-410, IRA-67 and IRA-900 manufactured by the Rohm and Haas corporation were purchased from the Sigma-Aldrich Canada. Hydrogen peroxide (30% w/w), sodium hydroxide, and hydrochloric acid were purchased from Fisher Scientific of Canada. High molecular weight polyethylene glycol (3500 g/mol) was purchased from Sigma Aldrich Chemicals of Canada. For pH measurement, an Accumet 915 pH meter was used. For all absorbance readings in colour measurement and enzyme activity assays, a Hewlett-Packard 8453 UV-Vis spectrophotometer was used interfaced with a Seanix computer in a Windows 95 environment. During enzyme immobilization, circulation was achieved via a Cole-Parmer peristaltic pump (1-100 RPM) controlled by a Masterflex drive. A Fisher vacuum pump was used to filter solutions in colour testing and to dry ion exchange resin.

Samples of kraft pulp mill effluent were taken from two separate mills in Alberta's boreal forest. One mill, Weldwood, employs an oxygen delignification system which lowers colour levels before leaving the mill, whereas the second mill,

Weyerhaeuser uses no colour reduction technology except peroxide addition at times of high colour events. For both mills, samples were taken from their final discharge, as well as the acidic and caustic sewers from their respective bleach plants. These bleach plants both operate with 100% chlorine dioxide (no elemental chlorine).

Wood chips were also used in the enzyme immobilization trials. The chips were white spruce chips, sent from the Weyerhaeuser mill. Each chip is approximately 4 cm in length, 3 cm in width and 1 cm thick. As the chips come from the stockpile at the mill, it cannot be determined with certainty the exact age of the chips or their moisture content.

## *3.2 Analytical Techniques*

The following assays and tests were used to determine the concentrations of the chemicals in the processes, activities of the immobilized enzymes and colour of the various wastewaters both before and after enzymatic treatment.

### 3.2.1 Soluble HRP Assay

To determine the activity of HRP in preliminary tests, a colourimetric assay was employed. This assay depends greatly on carefully controlling pH, temperature and substrate concentration. The colour-generating substrates used are phenol, hydrogen peroxide ($H_2O_2$) and 4-aminoantipyrine (AAP). The rate at which the colour forms is proportional to the amount of active enzyme present. The colour formed remains in solution and absorbs light at 510 nm with an extinction coefficient of

7100 $M^{-1}cm^{-1}$ (Buchanan 1996). Standard conditions for this assay use 10 mM phenol, 2.4 mM AAP and 0.2 mM $H_2O_2$, buffered to pH 7.4. Under these conditions a unit of activity (U) is defined as the number of micromoles of peroxide utilized in one minute.

The process of carrying out the soluble HRP assay is as follows. In a 1 mL semimicro-cuvette, the reactants are added in the following order:

- 500 µL 20 mM phenol
- 250 µL 9.6 mM AAP
- 100 µL 2.0 mM $H_2O_2$
- 50 µL 100mM sodium phosphate buffer at pH 7.4
- 100 µL of enzyme sample

Immediately after the enzyme is added, the cuvette is inverted and placed into the spectrophotometer. The spectrophotometer is programmed to read the absorbance of light at 510 nm every 6 seconds for one minute. The change in absorbance is used to calculate the activity of the soluble enzyme according to the following formula (Buchanan 1996):

$$SA = \text{Slope (AU/min)} / 7100 \text{ (AU·L/mol)} \cdot 10^6 \text{ (µmol/mol)} \cdot 1\text{L}/1000\text{ml} \quad (3.1)$$

where SA = Sample Activity in µmol/min·mL and AU = Absorbance Units.

The rate at which the colour is produced in the soluble HRP assay is linear with time for the period in which peroxide levels are in excess and phenol is being removed. As HRP acts very quickly, this linear period can be very short, before substrate depletion becomes rate limiting. The duration of the assay for soluble HRP

was 1 minute. Each time a new stock solution was prepared the solution was tested for its activity. In order to determine the linearity of the data, its $R^2$ coefficient was checked and seen to be greater than 0.95.

### 3.2.2 Immobilized HRP Assay

In order to determine the activity of the enzymes immobilized on the different media used in this investigation, a reliable method needed to be developed. The method used by Peralta Zamora and co-workers (1998) of taking the soluble HRP activity of the filtered fractions after immobilization was found to be unusable, as there was no significant difference between the activity of the original solutions and the activity of the filtered fractions after immobilization. However, this was not convincing evidence that no enzyme was being immobilized, due to the large amount of enzyme in the supernatant solution and the possibility that a small amount relative to the supernatant was immobilized. Thus, a new method was required that would be quick, reliable, and reproducible.

A colourimetric assay was developed to function in a manner similar to the soluble HRP assay. That is, the rate of colour formation in a reaction mixture of 10 mM phenol, 2.4 mM AAP and 0.2 mM $H_2O_2$ and an immobilized enzyme sample was measured at 510 nm. Measured amounts of medium (with enzyme attached) were added to 100 mL batches of this mixture and stirred at a constant rate. At regular intervals of 60, 90, 120, 150, 180, 240 270 and 300 seconds, 1 mL was removed from the reaction vessel and put into a semi-micro cuvette. 100 μL of catalase solution was added to the cuvette to quench the reaction by converting very

quickly any remaining peroxide to oxygen and water. This stops the reaction and prevents any more colour from forming in case the removed sample contains active enzyme sheared by the mixing of the reaction vessel. The colour intensity was then measured over time to calculate the amount of active enzyme immobilized on the medium per gram of medium added. The intervals and amounts of media added were chosen based on achieving a linear trace of colour production versus time, during which the assay is accurate; that is, when the formation of colour is directly proportional to the amount of peroxide being used up by the enzyme. The peroxide and other reactants are in excess throughout the assay and the rate of colour development is proportional to the enzyme activity.

### 3.2.3 Pulp Mill Effluent Colour Testing

Pulp mill effluent colour was determined via a standardized test. This test is widely used by members of the Canadian Pulp and Paper Association and is accepted as the industry standard. It is a colourimetric procedure which compares absorbance of sample versus an accepted standard at a predetermined pH and wavelength. The sample is filtered through a glass pore filter (Fisher G8 grade, cat. number 9-804-70D), and adjusted to pH 7.6 with HCl or NaOH. The samples are then filtered again, this time through a membrane filter (Millipore GT Type, Fisher cat. number GTTP 037 00) and adjusted to pH 7.6 again. The absorbance is measured on a visible light spectrophotometer at 465nm, and compared to the absorbance (Abs) of a cobalt chloroplatinate colour standard solution. The standard solution is defined as

500 platinum cobalt colour units (pccu or cu). Sample colour is then calculated via the following formula:

$$\text{Sample pccu} = (\text{Abs of Sample})_{465}/(\text{Abs of Std})_{465} \times 500 \qquad (3.2)$$

where Abs = Absorbance and Std = Standard.

## *3.3 Experimental Techniques*

### 3.3.1 Sample Preparation

Colour removal trials were conducted on a variety of samples from two kraft pulp mills in Alberta's softwood boreal forest. Final effluent was used from both mills without dilution or pH adjustment.

In the case of the Weyerhaeuser effluent, the sample was taken as a grab sample from the effluent stream of the aerated stabilization basin. The Weldwood final effluent samples were taken as grab samples from the effluent clarifier prior to secondary treatment. Final effluent samples from both mills were shipped to Edmonton via truck and stored at 4°C. Bleach plant effluents were taken from the alkaline and caustic sewers from the bleach plants of the mills. These bleach effluents were also stored at 4°C. All sampling and storage was done according to mill procedures to ensure the samples were representative of regular mill conditions.

Bleach plant effluent samples were prepared by mixing acidic effluent from chlorine dioxide bleaching steps with alkaline extraction effluents until the resultant sample was pH 6.8, which corresponded to the pH of the final effluents. This step

was taken to adjust the pH into the range where HRP is most active and the results could be better compared with the final effluent results.

### 3.3.2 Immobilization of HRP

Immobilization of the HRP enzyme on the support media was accomplished via a simple contact process. The support media were placed in a beaker in an aqueous solution of soluble enzyme and phosphate buffer at pH 7.4 and mixed at 4°C for a period of 20 hours. According to the method used by Peralta Zamora (1998) the temperature of 4°C is the optimum temperature for HRP immobilization on ion exchange resin. In order to ensure saturation, the soluble enzyme was used at high concentrations. As a result of this method, the ion exchange resin granules were broken apart. The method of immobilization was then altered to reduce the effect of shearing forces due to the mechanical mixing in the beaker. Instead, a simple chromatography column with an inner diameter of 4 cm and a length of 40 cm was used with a small peristaltic pump. A small mesh screen at the bottom of the column kept the resin from escaping. The enzyme solution was circulated through the media in the column via the pump. This was done to ensure contact between the enzyme and the media, while keeping shearing forces to a minimum.

After the twenty hour immobilization period, the media were removed from the column and washed with deionized water on a vacuum filtration apparatus. This was done in order to remove any soluble enzyme adhering to the media but not immobilized. After washing, the media were dried in air on the vacuum filtration apparatus until maintaining a dry appearance. As the resins used as media are

largely comprised of water, they were not dried further or in a dessicator. This air drying was simply to remove any adhering water that may contain soluble enzyme and give more accurate values when measuring specified masses of resin/enzyme.

### 3.3.3 Resistance to Shearing Forces Test

Another factor used in determining which was the best medium to use for the HRP immobilization was the ability to withstand liquid shear. If a flow-through reactor were to be designed to use this immobilized enzyme technology, the medium would need to be very shear-resistant. While it is not possible to give shearing forces a specific quantitative value, it was relatively easy to assess which of the media tested released the least active enzyme when subjected to liquid shear. Each medium, after immobilization, was washed and dried. The immobilized enzyme and resin were placed in solutions of phosphate and carbonate buffer and allowed to sit for 10 minutes and then the supernatant liquid was assayed for soluble HRP. It was found that the phosphate buffer caused the enzyme to desorb from the resin without any stirring, whereas the carbonate buffer did not cause dissociation. A measured amount of 10 g of resin/enzyme was added to 100 ml of carbonate buffer solution, and was stirred for a period of 10 minutes at level 3 of a Fisher magnetic stirrer with a 1 inch stir bar. These levels were chosen arbitrarily to give a moderate dose of shear, similar to what a flow through reactor may experience. Each test was done with the same magnetic stirrer, set at the same level. Every effort was made to ensure the conditions were identical for each test. After the ten minutes had elapsed,

the media with the enzyme were filtered and a sample of the supernatant was withdrawn and assayed for soluble enzyme.

### 3.3.4 Colour Removal

Colour removal trials were conducted as a three level, three factor, full factorial design experiment in duplicate. Each trial consisted of a 100 mL batch of one of the effluent streams and a set level of medium with immobilized enzyme, polyethylene glycol (PEG) and hydrogen peroxide. The design of the factorial experiment is below in Table 3.1:

**Table 3.1: Factorial Experimental Design**

| | 1 | 2 | 3 |
|---|---|---|---|
| Trial | PEG Level | $H_2O_2$ Level | Enzyme/Resin Dose |
| 1 | - | - | - |
| 2 | 0 | - | - |
| 3 | + | - | - |
| 4 | - | 0 | - |
| 5 | 0 | 0 | - |
| 6 | + | 0 | - |
| 7 | - | + | - |
| 8 | 0 | + | - |
| 9 | + | + | - |
| 10 | - | - | 0 |
| 11 | 0 | - | 0 |
| 12 | + | - | 0 |
| 13 | - | 0 | 0 |
| 14 | 0 | 0 | 0 |
| 15 | + | 0 | 0 |
| 16 | - | + | 0 |
| 17 | 0 | + | 0 |
| 18 | + | + | 0 |
| 19 | - | - | + |
| 20 | 0 | - | + |

| | | | |
|---|---|---|---|
| 21 | + | - | + |
| 22 | - | 0 | + |
| 23 | 0 | 0 | + |
| 24 | + | 0 | + |
| 25 | - | + | + |
| 26 | 0 | + | + |
| 27 | + | + | + |

In Table 3.1, the (-) symbol represents the low level tested of the three factors: 0 mg/L PEG, 1.16 mM peroxide and 5 g resin/enzyme dose in the 100 mL batch tests. The (0) represents the intermediate level of the three factors: 200 mg/L PEG, 5.8 mM peroxide and 15 g of HRP/resin. The (+) symbol represents the high level tested of the three factors: 400 mg/L PEG, 11.6 mM peroxide and 30 g of HRP/resin.

Once the HRP/resin, the peroxide, and the PEG were added to the reaction vessel, the vessel was left to be stirred gently with a magnetic stirrer at 25°C for a period of 20 hours. The period of 20 hours was chosen to ensure enough time was available for the HRP to remove all the colour it could, as done by Peralta Zamora *et al.* (1998). After the 20 hours had elapsed, the resin was filtered out and the effluent was tested for colour according to the method described in section 3.2.3. The used, filtered resin was again subjected to the immobilized enzyme assay to determine if any active enzyme remained immobilized after the colour removal trial.

In addition to the colour trials, a series of method blank trials were performed. It is known that some ion exchange resins are capable of adsorbing large organic molecules. Thus, it is possible that a significant portion of the colour removed in the trials was removed by the resin itself, and not specifically by the action of the enzyme. In order to determine this, method blanks were performed: these consisted of HRP without resin and resin without HRP. For the resin only

trials, no hydrogen peroxide was added and the amount of resin added corresponded to the three levels used in the colour removal trials: 5g, 15g, 30g.

# 4 Experimental Results

The first experimental portion of this study involved testing the soluble HRP assay to determine activity, as described in Section 3.2.1. This first portion was to establish that the enzyme was indeed active and its activity could be measured reliably. The next step was to immobilize the enzyme. Once immobilized, its activity was determined via the method described in section 3.2.2. The best medium was found to be IRA-400, based on activity retained and resistance to the shearing force test. The IRA-400 was then used to test colour removal from kraft mill final effluent in a factorial design experiment which tested the effects of enzyme dose, peroxide level and PEG level. Then, once optimum conditions were established, colour removal tests were performed on a weaker colour kraft effluent, as well as a combined bleach plant effluent from each of the two mills.

## *4.1 Immobilized Enzyme Assay*

Horseradish peroxidase enzyme was immobilized on each of four ion exchange resins. The results for each individual medium tested are listed below. All immobilization was performed with high concentration HRP stock solution, tested via the soluble enzyme assay at 250 units/ml.

### 4.1.1 IRA 900

This resin is a macroreticular type 1 polystyrene strongly basic anion exchanger which contains quaternary ammonium groups. It contains large pore sizes, forming

a sponge-like matrix which is capable of removing large size soluble organic particles. It was used in the $Cl^-$ form. As can be seen in Figure 4.1, the colour production in the assay was well within the linear period; therefore, it can be assumed that the slope of the line can be used to determine the activity of the enzyme immobilized to the resin. The total units of activity are then divided by the mass of the resin used for a result in active units per gram of resin.

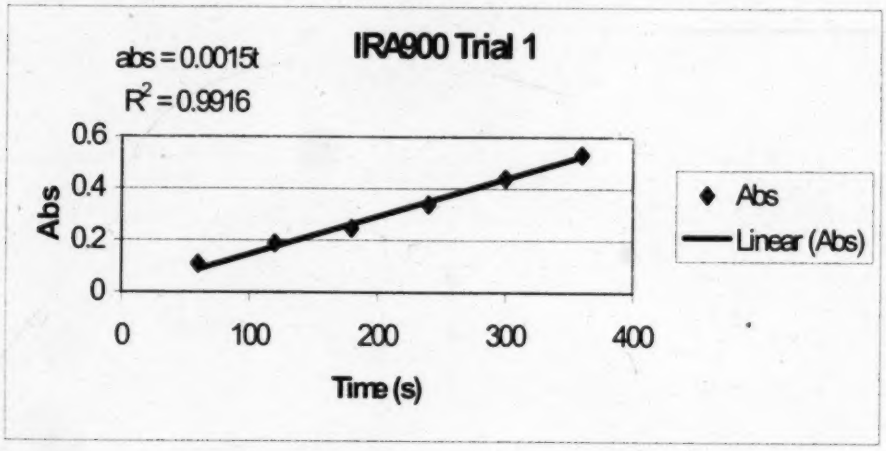


**Figure 4.1. Colour Production in the IRA 900 Assay.**

Using Equation 3.1 to calculate, the average activity retained on the IRA 900 resin was 1.25 units/gram of resin with a standard deviation of 0.14 units/gram over 6 trials. The resin beads absorbed the colour produced in the assay in the trials with IRA 900.

### 4.1.2 IRA 67

Unlike IRA 900, IRA 67 is a weakly basic anion exchanger. It has a crosslinked acrylic divinylbenzene copolymer matrix. The functional ion in IRA 67 is a tertiary amine. It was used in the free base form. Figure 4.2 shows the best, that is, most linear trial found for IRA 67.

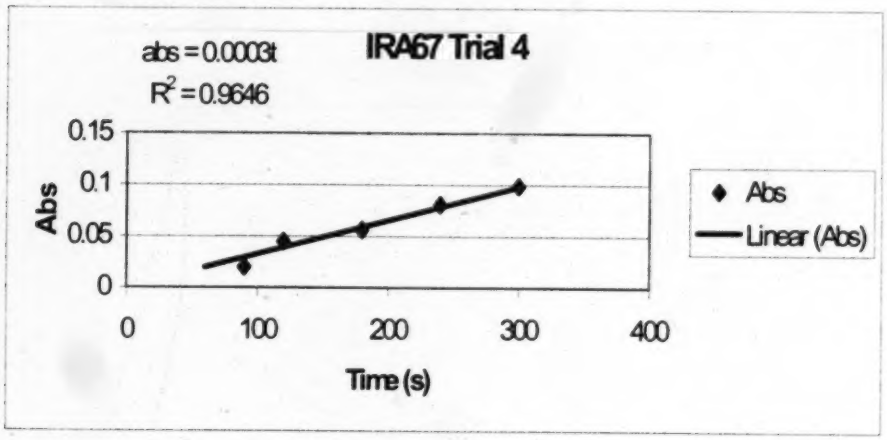


**Figure 4.2. Colour Production in the IRA 67 Assay.**

This resin did not immobilize much active enzyme. Using Equation 3.1, IRA 67 averaged only 0.08 units/gram of resin tested with a standard deviation of 0.02 units/gram over 6 trials. The resin beads did not change colour during the assay. As is shown in Figure 4.2, even after 300 seconds little colour change was observed, indicating that the amount of active enzyme immobilized on the IRA 67 resin was very small.

### 4.1.3 IRA 400

It was found by Peralta Zamora *et al.* (1998)that IRA 400 in its hydroxide ($OH^-$) form was an excellent medium for immobilizing active HRP. The hydroxide form of IRA 400 is no longer available commercially. However, it was available in its chloride form. According to the manufacturer's specifications, the chloride form may only be converted to the hydroxide form to a maximum of approximately 30%. One of the research goals of this project was to keep all materials and methods as low cost as possible, using only materials that are commercially available. Thus, the chloride form was tested. This resin is a strongly basic gel anion exchange resin. The functional group is a quaternary ammonium group and the matrix is a polystyrene divinylbenzene copolymer. Below in Figure 4.3 is an example of the IRA 400 assay.

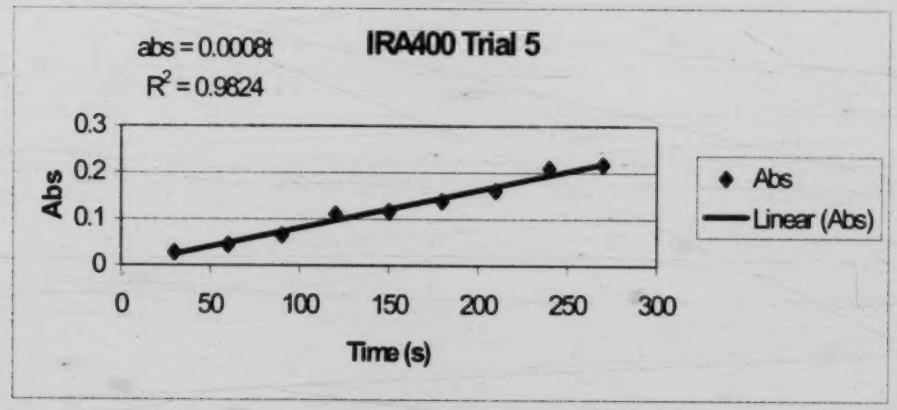


**Figure 4.3. Colour Production in the IRA 400 Assay.**

This resin retained an average of 1.0 unit/gram of resin, calculated via Equation 3.1. The standard deviation over 6 trials was 0.4 units/gram, indicating a larger range of values. As was the case with the IRA 900 resin, the beads either absorbed or adsorbed the red colour produced in the assay. As the resin beads contain water within them, it is uncertain whether the colour has been adsorbed or absorbed.

### 4.1.4 IRA 410

The fourth and final ion exchange resin tested was the chloride form of IRA 410. It is another strongly basic anion exchanger that has type 2 quaternary amines as its functional group in a crosslinked styrene divinylbenzene copolymer matrix. In Figure 4.4, an example of the colour production during an IRA 410 assay is shown.

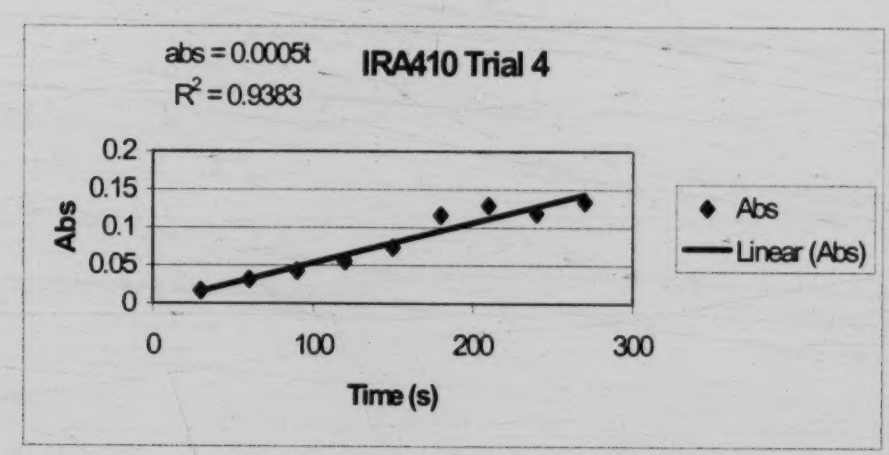


**Figure 4.4. Colour Production in the IRA 410 Assay.**

The IRA 410 resin did not yield results as repeatable as IRA 400 or 900. However, the assay graphs are mostly linear. The average activity retained on the IRA 410 was 0.23 units/gram of resin with a standard deviation of 0.12 units/gram.

### 4.1.5 Wood Chips

Wood chips were also tested as a low cost option, to see if this material had potential to be a medium that could immobilize HRP. Screened spruce chips were obtained from one of the pulp mills and put through the same procedure as the resin for immobilization. Figure 4.5 shows an example of colour production in the wood chip assay.

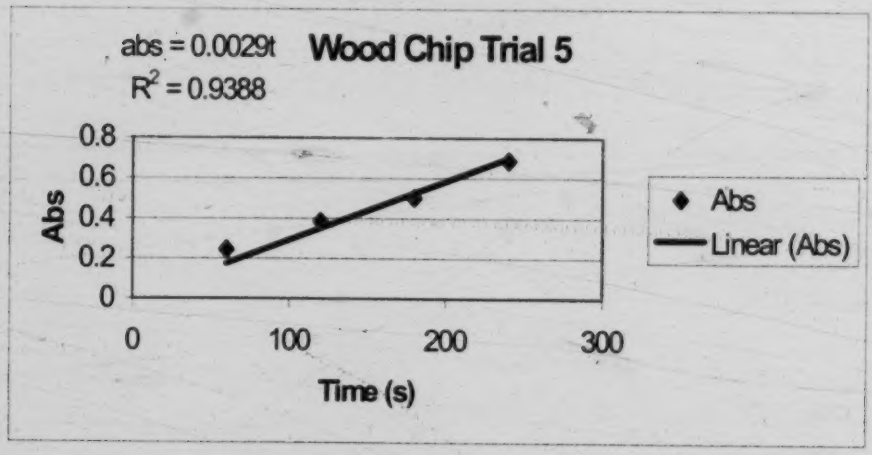


**Figure 4.5. Colour Production in the Wood Chip Assay.**

The wood chips performed quite well in the immobilized enzyme assay, averaging 0.4 units/gram of wood with a standard deviation of 0.1 units/gram. However, at the time of this investigation it was not possible to determine how much

of this activity is immobilized enzyme and how much is soluble enzyme that had been absorbed into the interstitial spaces in the wood. Whereas the wood chips did in fact show retention of active enzyme, it can not be stated with certainty that this method immobilized the enzyme. If HRP on wood chips is to be investigated further, it will need a different method of immobilization, or a different assay must be developed to determine reliably the amount of immobilized enzyme.

### *4.2 Fluid Shear Tests*

The amount of immobilized enzyme is not the only important criterion in finding a suitable medium for immobilizing HRP. If this technology were to be used in pilot scale or plant scale, it would likely be in some type of flow through reactor. Thus, the enzyme must have good resistance to shearing forces of moving liquids. If the enzyme were to be easily sheared off, the remaining medium would not be useful, as no active enzyme would remain. Thus, each of the resins tested was also subjected to shearing forces, as described in section 3.3.3.

#### 4.2.1 IRA 900

The IRA 900 resin had shown the highest capacity for immobilizing active HRP, at 1.25 units/gram. However, once subjected to the fluid shear test, the supernatant liquid showed that an average of 117% of the active enzyme had been sheared off by the stirred liquid. It is possible to have a number greater than 100% here; the resin had immobilized an amount of HRP but only 1.25 units/gram were active. Thus, once subjected to shearing forces, the active and inactive enzymes had come free

from the resin. It can also be seen by this observation that the HRP was immobilized in some cases in an orientation that did not permit it to be active. The IRA 900 resin showed the highest capacity for retaining immobilized active enzyme but very poor performance in being resistant to shearing forces. Thus, the IRA 900 was not the medium chosen to do colour removal trials, as the colour removal trials involved constant stirring.

### 4.2.2 IRA 67

In the case of the IRA 67 resin, the fluid shear test showed a 41% removal by shear rate of the active enzyme immobilized to the beads in 3 of the 6 trials. In the other trials, results varied greatly, producing values of 17% and 83%. It should be noted that the total amount of active HRP immobilized was only 0.08 units/gram before the shear test. Thus, there was a very small amount of enzyme to deal with in the first place. Due to the very small amount of immobilization, coupled with a relatively high removal by shear, the IRA 67 resin was not chosen to perform colour removal tests.

### 4.2.3 IRA 400

The IRA 400 resin had the second highest average activity immobilized. When subjected to the fluid shear test it performed very well, with an average removal of only 1.63% of the active enzyme due to the stirring of the liquid. This was the best

combination of immobilization and shear resistance, and was therefore used in the subsequent colour removal tests.

### 4.2.4 IRA 410

The IRA 410 resin also performed relatively well in the fluid shear test, averaging 10.6% removal of the active HRP. However, since the average retention of active enzyme was only 0.25 units/gram it is clear that the IRA 400 is a better choice to use for the colour removal tests.

A summary of enzyme immobilization and shear resistance testing is contained in Table 4.1.

**Table 4.1. Summary of Immobilization and Fluid Shear Tests**

| Medium | Average Immobilization (units/gram) | Activity Retained after Shear test (units/gram) | Activity Released (%) |
|---|---|---|---|
| IRA 67 | 0.08 | 0.047 | 41.1 |
| IRA 400 | 1.0 | 0.98 | 1.63 |
| IRA 410 | 0.25 | 0.23 | 10.64 |
| IRA 900 | 1.25 | 0 | 116.8 |
| Wood Chips | 0.4 * | N/A | N/A |

*Assuming activity is immobilized and not interstitial fluids. Please see section 4.1.5.

## *4.3 Colour Removal Trials*

This section contains the results of the major experimental part of this investigation. After the enzyme had been immobilized by the IRA-400 ion exchange resin, the colour removal trials were performed. A three level, three factor experiment was carried out using factorial design to test the effects and interactions of PEG, hydrogen peroxide and enzyme/resin dose, as described in section 3.3.4. The main results are listed below. A full account of all results is available in Appendix B. It should be noted that typically, Weyerhaeuser effluent has a colour value of 850-1300 pccu.

### 4.3.1 Factorial Experimental Results

The full $3^3$ factorial experiment was conducted in duplicate and the average result was used for data analysis. With only a few exceptions, the replicates differed by 5% or less. A representation of the experimental design and averaged results of the replicate trials are shown below in Table 4.2. Original colour for the effluent was 1065 pccu.

**Table 4.2. Factorial Experiment Results**

| Trial | 1 PEG Level | 2 $H_2O_2$ Level | 3 Enzyme/Resin Dose | Average Colour Remaining (pccu) | Average Colour Removed (pccu) |
|---|---|---|---|---|---|
| 1 | - | - | - | 490 | 575 |
| 2 | 0 | - | - | 460 | 605 |
| 3 | + | - | - | 465 | 600 |
| 4 | - | 0 | - | 330 | 735 |
| 5 | 0 | 0 | - | 415 | 650 |
| 6 | + | 0 | - | 365 | 700 |
| 7 | - | + | - | 300 | 765 |
| 8 | 0 | + | - | 310 | 755 |
| 9 | + | + | - | 315 | 750 |
| 10 | - | - | 0 | 290 | 775 |
| 11 | 0 | - | 0 | 320 | 745 |
| 12 | + | - | 0 | 330 | 735 |
| 13 | - | 0 | 0 | 250 | 815 |
| 14 | 0 | 0 | 0 | 255 | 810 |
| 15 | + | 0 | 0 | 280 | 785 |
| 16 | - | + | 0 | 195 | 870 |
| 17 | 0 | + | 0 | 220 | 845 |
| 18 | + | + | 0 | 220 | 845 |
| 19 | - | - | + | 180 | 885 |
| 20 | 0 | - | + | 180 | 885 |
| 21 | + | - | + | 180 | 885 |
| 22 | - | 0 | + | 180 | 885 |
| 23 | 0 | 0 | + | 155 | 910 |
| 24 | + | 0 | + | 150 | 915 |
| 25 | - | + | + | 130 | 935 |
| 26 | 0 | + | + | 145 | 920 |
| 27 | + | + | + | 145 | 920 |

As can be seen in Table 4.2, the colour removal is highest for high values of the factors tested, suggesting that maximum removal has not been reached. Some high level HRP/resin dose trials yield greater than 85% removal of the total colour from kraft mill final effluent. At first glance, it appears the only significant factors are the enzyme dose and perhaps the peroxide dose that are contributing to colour removal.

Main effects and interactions are calculated from a $3^k$ factorial design by using Yates' Algorithm (Box and Draper 1987).

### 4.3.2 Effects and Interactions

In a $3^3$ factorial experimental design there are a total of 27 possible effects and interactions which can contribute to the results of the trials. Not only are there the three main effects, but all the possible first order interactions and even the second order interactions. That is, the interactions can include the squares of the main effects. Fortunately, Yates' Algorithm provides values for all the main effects and interactions and their mean squares. The mean square of errors was calculated by taking the sum of the squares of the difference between the replicate trials divided by $2^{k-1} = 4$. Using the ANOVA method, significance was determined by dividing the mean square of the effects by the mean square of error and comparing the result to the critical "F" value for a 95% confidence interval. If the result was greater than the critical F value for 24 degrees of freedom (1.89), the effect or interaction was deemed significant. The only factors that proved to be significant in this experiment were the enzyme/resin dose and the hydrogen peroxide dose. The PEG level was found to be insignificant in removing colour, as were all the combinations of first and second order interactions. Complete results for the steps of Yates' Algorithm and ANOVA results can be found in Appendix B.

### 4.3.3 Optimum Conditions

The optimum conditions for colour removal were determined from the factorial experiment on the final kraft effluent in order to test the colour removing potential on other waste samples. As both peroxide and enzyme/resin effects are positive in Equation 4.1, and the highest doses provided the best results, the high dose levels were used for the other effluent samples. That is, 11.6mM $H_2O_2$ and 30g of HRP/resin.

## *4.4 Use of Optimum Conditions on Other Wastes*

To determine at which point in the bleach process the treatment is most effective, colour removal from three other kraft effluents was tested. These included: a low colour final effluent from a different mill (Weldwood), a mixture of acidic and caustic effluent from the Weldwood bleach plant, and a mixture of "$D_o$" (acidic effluent from first chlorine dioxide bleaching step) and "$E_{op}$" (caustic effluent from oxygen/peroxide alkaline extraction step). As described in Section 3.3.1, the bleach effluents were adjusted to pH 6.8. All tests were performed in triplicate and the average result calculated. The results are listed below.

### 4.4.1 Weldwood Final Effluent

Final effluent from the Weldwood of Canada kraft pulp mill in Hinton, Alberta was tested under the conditions described above. Initial pH of the effluent was 8.8. The characteristics of this final effluent differ from the Weyerhaeuser effluent. For

example, the original colour of this effluent was only 420 pccu, as opposed to the 1065 pccu tested for the Weyerhaeuser effluent. The main reason for the difference is the presence of an oxygen delignification step prior to bleaching in this mill. This step is known to greatly reduce colour. After 20h at 25°C with 11.6mM $H_2O_2$ and 30g HRP/IRA-400, the final colour of the Weldwood effluent was 110 pccu, a 73% removal, or 310 pccu.

### 4.4.2 Weldwood Acid and Caustic Sewers

The combined wastes from the acidic and caustic sewers from the Weldwood bleach plant had higher colour levels than the Weldwood final effluent. This is because the bleaching effluents are the major contributors to pulp mill effluent colour. Initial colour of this stream was 590 pccu. Results of the colour removal test for this waste stream was 30 pccu, a removal of 95% of the colour initially present.

### 4.4.3 Weyerhaeuser Acid and Caustic Sewers

The combined acidic and caustic bleach plant effluent from the Weyerhaeuser mill contained the highest colour of all effluents tested. This mill does not use oxygen delignification, and the bleach plant is again the major contributor to final effluent colour. At this point the colour is concentrated, before the other process wastewaters dilute the colour before it enters the receiving water. The initial colour of this effluent was 1365 pccu. After treatment, final colour was 370 pccu, a removal of 73% of colour.

### *4.5 Immobilized Enzyme Assay to Test Residual Activity After Use*

After the enzyme/resin was used to remove colour from the Weyerhaeuser effluent a portion was saved and tested again for any remaining HRP activity. It was found that a small amount of enzyme activity remained, 0.03 units/gram of resin. Data from this trial and calculations can be found in Appendix B.

### *4.6 Tests to Determine Contribution to Colour Removal by Enzyme vs. Resin*

As described in Section 3.3.4, method blank trials were performed to determine the relative contributions to colour removal from the resin IRA 400 and the enzyme itself. Results from the blank trials are shown below in Table 4.3. It was found from these tests that the HRP enzyme was not removing any colour at all; the resin and peroxide were responsible for all the colour removal in the trials. At the 5g level the resin alone removed 590 pccu, the 15g level removed 780 pccu and the 30g level removed 840 pccu from the 1065 pccu Weyerhaeuser final effluent.

**Table 4.3. Results of Method Blank Trials in Weyerhaeuser Effluent**

| | **Amount of IRA 400 Resin (g)** | **Colour Removed (pccu)** |
|---|---|---|
| Resin Only | 5 | 590 |
| Resin Only | 15 | 780 |
| Resin Only | 30 | 840 |
| | **Amount of HRP (units)** | **Final Colour (pccu)** |
| HRP +$H_2O_2$ | 15.3 | 1169 |
| HRP +$H_2O_2$ | 153 | 1297 |
| HRP +$H_2O_2$ | 800 | 1234 |

These results show that the HRP enzyme is not effective in removing colour from the kraft effluent samples tested. In the cases where the HRP is added without resin, the final colour is actually higher than the initial value of 1065 pccu.

# 5 Discussion

This section discusses all the results and findings of the investigation, their significance and applicability. Directions for future research will also be discussed.

## *5.1 Method for Immobilizing HRP*

The method for immobilizing HRP (described in Section 3.3.2) utilized a closed loop and a peristaltic pump. This method was developed to prevent the resins from breaking down due to the force of mechanical stirring. The peristaltic pump was a good solution to this problem. It allowed the enzyme solution to be in continuous contact with the support media without breaking it apart. Also, fluid shear forces were kept to a minimum during immobilization to minimize partially adsorbed enzymes being removed before immobilization was complete. Immobilization was done at 4°C, as was done by Peralta-Zamora and co-workers (1998). It is unclear why immobilization is more effective at the lower temperature than it is at room temperature. There are no differences chemically in the resins or enzyme or buffer solution between the two temperatures. One possible explanation is that less thermal motion at the lower temperature lessens the shifting of the shape of the enzyme molecules, allowing it to become immobilized such that its active sites remain in the correct shape. Thus, the enzyme would become immobilized when it is in a state of reduced activity and would presumably regain the bulk of its activity when brought back to optimum temperature. It is already known that HRP is less active at this low temperature. For the temperature dependence of HRP, please see section 2.6.

### *5.2 Development of Method for Immobilized Enzyme Assay*

The method used to determine the activity of immobilized enzyme was developed during this investigation. No simple, low cost, reliable option was available at the time. Other options investigated (as mentioned in section 3.2.2) included differential activities in supernatant fluids after filtration, but this method was not applicable for small amounts of enzyme immobilized, nor would it indicate if any inactivation of the enzyme occurred during immobilization. The method developed in this investigation was a relatively simple procedure. All of the reagents used are readily available for purchase on the commercial market. The quantities used in a trial are not very large and at relatively low concentrations.

For the method to be considered useful in future investigations, it is also important that the results be reliable. This is best demonstrated by a good level of precision; that is, reproducible results. For each resin tested, there were six repetitions of each assay to establish precision. It was found that for the resins which immobilized the most HRP, that is, IRA 400 and IRA 900, the precision was higher than for the resins which immobilized less enzyme. This is expected, as the less quantity there is to detect, the more difficult it is to discern from background "noise" levels. For IRA 900, the standard deviation in the calculated activity was 11% of the mean value with a 95% confidence interval of 0.14 activity units per gram. For IRA 400, eight trials were performed with one outlier result which was discarded. The standard deviation of the calculated activity was 30% of the calculated mean value of immobilized activity with a 95% confidence interval of 0.28 activity units per gram. These values do not suggest that the developed method

is very precise; the variance in results may be deemed unacceptable in a more detailed investigation. However, in this investigation, the method was able to distinguish which resins immobilized the most enzyme with a good level of confidence based on the differences in the average amounts retained for each resin tested.

The duration of the trial is only a few minutes, with little preparation time of the reagents. This method is fast, easy and low cost, which was one of the main objectives of this investigation. The method is reliable, in that it gives a definite indication as to whether or not enzyme has been successfully immobilized, and a good indication of how much. Whereas it is true that there was an amount of variance in the results it was still clear which resins retained the most active enzyme. For these reasons the objective has been met; a reliable, low cost and simple method to determine retained activity of horseradish peroxidase has been developed.

### *5.3 Comparison of the Resins*

As discussed in Section 2.6.3.4, the mechanism of immobilization which occurred in this investigation included both ion exchange and physical adsorption.

The experimental results showed that the four ion exchange resins retained disparate levels of activity. All four resins were basic anion exchangers. The properties of the resins will be compared below.

IRA 67 is a weakly basic exchanger. It uses a tertiary amine as its functional group in a crosslinked acrylic divinylbenzene copolymer matrix. The pore spaces are smaller in the IRA 67 resin than in the others. It was the only weakly basic resin

tested. It retained the least amount of active HRP, much less than the others. The most obvious difference is that the exchanger is weakly basic, rather than strongly basic with smaller pore sizes. As these are the main physical differences between the resins, it is likely that these are the main contributing factors to the lack of retention of active enzyme.

IRA 410 is a strongly basic exchanger, which, like IRA 400, has quaternary amines as functional groups. Like IRA 400 and IRA 900, it has a crosslinked polystyrene divinylbenzene copolymer matrix as its' structure. However, IRA 410 retained much less active enzyme than IRA 400 and IRA 900. It is most likely that the weaker affinity for the HRP is due to a smaller pore size which is unsuitable for HRP adsorption and ion exchange. The enzyme molecules may not fit the pore sizes well at all, or else they fit poorly, such that the majority of the active sites are not able to function properly.

IRA 400 resin is also a strongly basic anion exchanger. It also has a quaternary ammonium as the functional group. The greater affinity for the HRP is most likely due to larger and more correctly fitting pore sizes which allow the HRP to remain securely immobilized while keeping the enzyme's active sites available for reaction; that is, it may still undergo the transformation to Compound I as seen in Equation 2.1.

IRA 900 resin has the same structure and functional group as IRA 400. It was able to retain more active HRP but lost all of the retained activity during the shear test. It is most likely that the pore sizes are large enough to immobilize the enzyme, yet offer no protection to the shearing force of the moving fluid, causing

rapid desorption. The results of the shear test showed that not only was all of the active enzyme was removed, but also that some enzyme had been immobilized in such a manner that it was inactive when adsorbed.

From the testing performed, the best resin for immobilizing and retaining active HRP was IRA 400. IRA 400 had the properties found to contribute to good affinity and resistance to shear for HRP: correct pore size for immobilization which also offers protection from shearing force, sponge-like polystyrene matrix, quaternary amine functional group in a strongly basic anion exchange resin.

### *5.4 Shear Testing*

The method used to test resistance to shearing forces was also developed during this experiment. The concept was simple: apply a "dose" of fluid shear to the immobilized enzyme on the support medium and determine how much activity is removed. While it is impossible to specifically calculate an amount of shear applied, every possible step was taken to ensure that each trial involved the same amount of shearing force applied. These steps were using the same equipment at the same power levels while mixing.

The results of this test showed very good reliability; that is, a high level of precision with reproducible results. Over six trials for each resin tested, results for removal of active enzyme varied only slightly. Standard deviation was never more than 10% of the mean calculated value (See Appendix) except for IRA 67, which retained so little enzyme that the detection limit of the assay was approached and measurements were unreliable. Over six trials, the calculated mean removal of

active enzyme from IRA 400 was 1.63% of the total 1.0 units/gram, or 0.0163 units per gram removed. The standard deviation was 0.16% for these results. Clearly this test is reliable for testing resistance to shearing forces. It is a good, useful method applicable in future investigations involving immobilized enzymes.

### *5.5 Colour Removal*

Previous studies, such as that of Peralta Zamora *et al.*(1998) (see section 2.5.5) have used immobilized HRP to remove colour from pulp mill effluent. This colour removal is achieved by the removal of chlorophenols and other aromatics which HRP acts on. These chromophoric bodies are polymerized until they become insoluble and settle out of the solution. In the previous studies both soluble and immobilized HRP were effective in removing colour forming bodies from the effluents tested. In this investigation it was found that the enzyme itself was not responsible for any of the colour removed in the trials. Instead, the colour was removed by the ion exchange resin itself. (Please see Table 4.3) This conclusion was made from comparing resin blanks and method blanks with the actual trial results.

It was found that a slight contribution to colour reduction was due to the hydrogen peroxide; this was an expected result, as hydrogen peroxide is a strong oxidant and is known to break down colour bodies. Peroxide is sometimes added to pulp mill effluent as a measure to reduce colour before discharge to the receiving water.

The resin IRA 400 removed a great deal of colour, near 27 pccu per gram of resin from final kraft effluent. In the product descriptions of IRA 900 and 400 from the manufacturer, it is stated that the large pores, sponge-like matrix and strong basicity allow the resin to remove larger soluble organics from solution. An article by Fitch (1980) describes cases where ion exchange technology was used to remove pollutants and colour from bleach plant effluent. The study in question utilized pilot scale experiments with 60 $m^3$ columns containing 13.1 $m^3$ of ion exchange resin. Flowrate was fixed at three bed volumes per hour. It was found that weakly basic ion exchange resin was capable of removing 70% of colour of high colour bleach plant effluent. Values for colour at the influent to the column ranged from 12000-14000 colour units. Fitch (1980) stated that soluble organics with strongly ionized molecular groups will be strongly attracted to ion exchange resin and will bond very tightly. These organics with strongly ionized groups will have such an affinity for the resin that they will even displace organics with more weakly ionized groups. Thus, whereas it is not a widely used technique, there is already some use in the industry of ion exchange technology to remove colour.

This technology has not yet been tried in the mills in Alberta. Each pulp mill's effluent is different, and colour forming bodies vary greatly. However, from the results of this investigation, clearly there is a great potential for further study. The ion exchange resin IRA 400 was capable of removing a great deal of colour from final effluents and bleach plant effluents. The resin should, in the future, be tested for its potential to remove colour from black liquor. If the resin is capable of removing colour and contaminants from black liquor, it may be very useful in

preventing detrimental effects on the environment due to black liquor spills, as well as maintaining good environmental performance of the mill. There are a large number of different ion exchange resins to be tested for their potential to remove colour and other contaminants. There may be cost effective solutions to pulp mill colour and contaminant issues that can be solved with ion exchange technology waiting to be discovered. Moreover, there are many other ion exchange resins readily available that may potentially remove colour even better than IRA 400.

The next question to be answered in further research with IRA 400 is how tightly bound the colour bodies are to the resin. Is it possible to remove the colour and reuse the resin? Ideally, it would be possible to remove the attached colour, in a step similar to a regeneration step, that would allow the resin to be used over and over. If this is possible then perhaps ion exchange technology is an attractive option for industry, which will be facing tougher and tougher colour discharge limits in operating permits.

### *5.6 Colour Structure*

The major question arising from the experimental results of this investigation asks why the HRP did not remove any colour. HRP has been used in numerous studies to specifically remove colour from both bleach plant effluent and final mill effluent. It was expected that the effluents tested would also have their colour reduced by HRP.

As stated in Section 2.1, colour forming bodies are thought to be made up largely of chlorophenols, quinones, aromatic ketones, high molecular weight compounds with many aromatic rings. From previous studies and an understanding

of how HRP removes substrates by forming radicals, it would be expected that HRP would remove colour. Since no colour was removed by the HRP, it may be the case that at the least the bulk of the colour forming bodies in the effluents tested do not contain phenolic compounds, such as chlorophenols. This would contradict what was previously believed about pulp mill colour.

A noticeable contradiction with the work of Peralta Zamora *et al.* (1998) concerns the adsorption of colour bodies to the resin support. In the Peralta Zamora *et al.* investigation it was found that for kraft final effluent a small portion of the decolourization could be attributed to adsorption to the resin support, and for the E1 (first alkaline extraction bleach plant) effluent and the combined bleach plant effluents there was no contribution due to adsorption by the resin. In this investigation the resin adsorbed colour bodies from each type of effluent. This difference is further indication that it is likely that the colour forming species in the Brazilian study are different than the colour forming species in the Alberta kraft mill effluent tested in this investigation.

A possible explanation for the difference in colour body structure in the Alberta mills and previous studies is in the bleaching sequence. Both of the mills tested in this investigation use an elemental chlorine-free bleaching sequence. Elemental chlorine-free bleaching is a relatively new process which uses chlorine dioxide bleaching steps rather than elemental chlorine. Alberta mills were converted to elemental chlorine free bleaching in the early 1990's in order to prevent the release and formation of dioxins and furans such as 2,3,7,8-tetrachlorodibenzodioxin in the receiving waters. Each of the previous studies cited which used HRP to

remove pulp mill effluent colour were performed on effluent from mills which still used at least one elemental chlorine bleaching step. There is very little information available which compares colour structure from elemental chlorine bleaching processes with colour structure from elemental chlorine free bleaching processes. However, Kemeny and Banerjee (1997) wrote that substituting chlorine dioxide for chlorine increases the population of lower chlorophenol congeners. Moreover, these lower congeners are much more biodegradable than more highly substituted chlorophenols. If these chlorophenols made up the colour, and were in fact highly biodegradable, colour would be lowered across the effluent treatment system. This is not the case at either of the test mills. Thus, the colour is not made up of chlorophenols, at least not lower congeners, in any large part.

It is important to understand why the HRP did not remove any colour. It is likely that the elemental chlorine free bleaching process produces colour forming bodies that are not substrates for HRP. One possible explanation is that the colour forming bodies produced in these processes are too large for the enzyme to handle, or cannot fit the enzyme's active sites due to its shape or other steric factors. This is a highly unlikely explanation, as HRP only needs one functional group on one end of a large molecule to form a free radical which may then go ahead and polymerize. Moreover, HRP continues to form larger and larger free radicals until the polymers formed are so large that they cease to be soluble. Thus, size and shape of the colour bodies should not matter in treatment with HRP. All HRP needs to work is a single functional group with which it may create a free radical.

To gain an understanding of why HRP works on certain substrates and not on others, the free radicals formed when HRP catalyzes removal of phenols and chlorophenols were studied. When the radical is formed, a proton is removed, leaving a free radical. When two free radicals meet, the extra electron on each form a bond and a larger molecule is created. Free radicals are almost always intermediates in reactions as they are usually highly unstable (Carey and Sundberg 1993). However, some structural features in organic molecules increase stability of free radicals. When free radicals without any stabilizing structures form in organic molecules, they will rapidly disproportionate or they will immediately acquire a proton.

The most basic substrates of HRP are phenols and chlorophenols. Upon study of these substrates it was discovered that the aromatic ring structures present would provide stabilization during the formation of the free radical species. When the activated HRP comes in contact with the phenolic substrate, it will remove a proton and form the free radical. As this happens, the structure of the aromatic ring stabilizes the free radical just enough to allow it to exist as a free radical long enough to come in contact with another nearby free radical and dimerize, rather than taking a proton back.

The stabilization provided by the aromatic ring, which can also exist in aromatic amines, quinones, and many other structures, is achieved by the delocalization of the excess negative charge. Aromatic rings are especially good at delocalizing charge due to their conjugated double bonds, flat ring structure and especially their pi-bonding orbitals. These pi bonds make aromatics very stable.

Large clusters of aromatics in large soluble organics can delocalize excess negative charge greatly. Charge delocalization is achieved by a movement of the unpaired bonding electron through the pi orbitals. Distributing the negative charge across the aromatic ring makes the radical more stable (Carey and Sundberg 1993). Phenols and aromatic amines even further increase the potential to delocalize negative charge. These compounds have substituted strongly electronegative oxygen or nitrogen atoms for hydrogen atoms attached to the carbon atoms which make up the aromatic ring structure. Because electronegative atoms have a much greater affinity for electrons, the delocalization of charge is even greater and the free radical is more stabilized. To illustrate the delocalization of negative charge, organic chemists draw resonance structures. Resonance structures are analogs of the molecule with excess negative charge, showing the possible movements of the electrons and the potential areas where the negative may exist.

As a general rule, the more resonance structures possible for a free radical, the more stable it will be. It is easy to see how a large molecule with several aromatic rings would make a stable free radical. Thus, HRP acts by forming free radicals that are just stable enough to not immediately abstract a proton or react with oxygen, but to dimerize with a nearby free radical. As the concentration of free radicals becomes greater, the chance of forming dimers becomes greater. These dimers become larger and larger until they are no longer soluble and settle out. Phenols, chlorophenols, aromatic amines and many other structures provide just enough stability to dimerize, making them good substrates for HRP. Recall that HRP is one of the least specific peroxidases; it catalyzes the removal of many soluble organics from solution. It is

the opinion of the author that the stabilizing effect of the structures is what determines whether HRP will catalyze the removal of a colour causing chemical.

In this investigation, the enzyme HRP did not remove any colour. The enzyme's activity had been tested, and there was hydrogen peroxide present to activate it. The temperature and pH of the effluents during the trials fell well within the known ranges for effective HRP activity. Clearly the colour forming bodies are simply not substrates that HRP is effective on. HRP has been used in the past to remove colour from pulp mill effluent; however in this case it did not. The best possible explanation as to why follows. The main difference in the effluents that were decolourized by HRP and these which were not was the bleaching process used. It is possible that substituting chlorine dioxide for chlorine bleaching produces a different set of colour forming compounds. This colour is made up of largely aliphatic (not aromatic) compounds which are not capable of delocalizing a charge, and thus, are unable to stabilize a free radical. Because the free radical is so unstable, the HRP is either unable to successfully remove a proton or else the radical immediately gains another proton back. Thus, the HRP is not effective at removing the compounds, and no colour is removed.

It is also possible that the reason for the difference in the effluent components may be due to differences in climate, geology and the makeup of lignin in different types of wood. None of the previous studies used northern boreal forest softwood. It is possible, theoretically, that the structure of lignin in other climates with differing soil geology varies so greatly that the compounds solubilized during the pulping process are different. If this is the case, it could explain why the colour

can be removed by HRP from effluent in Brazil by Peralta-Zamora (1998) but not in northern Alberta.

Because the precise structure of colour compounds is not specifically known, it can not be stated with certainty if this given explanation is correct. However, it is the opinion of the author that it is the most likely scenario that between different wood and different pulping and bleaching processes, different colour causing compounds are produced in kraft mills in Northern Alberta and these compounds are not affected by the enzyme horseradish peroxidase.

In comparing the amount of colour removed versus the increasing amount of resin added, it was found that there was not a linear increase of colour removed as resin mass increased. The graph illustrating this is in the Appendix. The graph must pass through the origin, as no resin added will result in no colour removal. When viewing the graph with a smoothed line, the graph seems to indicate that a maximum amount of colour removal can be reached. Thus, increasing the resin dose will not result in total colour removal. The most logical conclusion from this data is that not all of the colour bodies are removable by the resin IRA 400. The resin will adsorb certain colour bodies but not others. Of the certain colour bodies the resin is able to remove, it will remove more of them as resin dose increases. Since the compounds that make up colour in pulp mill effluent vary, it is likely that some colour forming bodies are not suitable for removal by IRA 400. These remaining colour bodies may simply just be of the wrong structure to bind to the resin.

### *5.7 Comparing the Effluents*

The last objective of this investigation to be answered is upon which effluent is this new technology most effective. From this information, it can be determined at which point in the pulping process a reactor would best be placed, if put online.

In analyzing the data, it was found that the largest amount of colour removed per gram of resin was for the high colour bleach plant effluent from the mill which did not use oxygen delignification (Weyerhaeuser). For this effluent, a removal average of 95% was recorded. The next highest amount was for Weyerhaeuser final effluent, 88%, which was also the next highest colour waste tested. The Weldwood bleach plant effluent and final effluent, respectively had removals of 74% and 73%. The full results to these trials are located in Section 4.4. The resin removes the most colour from the high colour wastes. Thus, the highest colour stream makes the best candidate for this treatment. As discussed in Section 5.6, the resin does not remove all the colour present in the sample. It is possible that the colour bodies removed in the oxygen delignification process are the same colour bodies removed by the resin IRA 400. If this is in fact the case, then perhaps ion exchange technology could be investigated as a low cost alternative to oxygen delignification.

Because the resin removes the most colour, and is thus the most efficient, from the high strength colour streams, it would be most prudent to use this technology on the bleach plant effluent.

# 6 Conclusions

This investigation has led to the conclusions outlined below.

## *6.1 Method for Determining Activity of Immobilized Enzyme*

After exploring other possibilities, a method was developed to determine the amount of active enzyme which could be immobilized on a solid support. This method involved a small batch assay that would change colour over time. At known time intervals, small samples were removed and the reaction stopped for a colourimetric determination of immobilized enzyme activity. This method proved to be reproducible and reliable for resins which retained active enzyme.

## *6.2 Comparison of Ion Exchange Resins*

Four basic anion exchange resins were compared for their capacity to retain active enzyme and hold on to it when exposed to shearing forces. It was found that the strongly basic resin Amberlite IRA 400 performed the best of those tested, retaining 1.0 unit of activity /gram and showing excellent resistance to fluid shear.

## *6.3 Colour Removal*

The immobilized enzyme was tested for its capacity to remove colour from pulp mill effluent. In order to determine maximum removal conditions, a $3^3$ factorial design experiment was performed, varying levels of polyethylene glycol, hydrogen peroxide and enzyme/resin dose. It was found that the polyethylene glycol had no

significant effect on colour removal, neither did any of the calculated interactions. The main effects of hydrogen peroxide dose and enzyme/resin dose were found to be significant via Yates' Algorithm. Further investigation found that it was in fact the resin itself and not the enzyme which was responsible for colour removal. The colour possesses strong ionic groups which facilitate the colour to bind to the resin.

### *6.4 Colour Structure*

Traditionally, the structure of colour forming bodies was thought to be largely phenols, and other aromatics. These compounds have been successfully removed in the past by HRP. However, in this investigation the HRP was ineffective in removing any colour; thus, the compounds making up the colour in the effluent tested must not be phenols and aromatics. The best possible explanation is that the colour bodies formed lack the capacity to form a stabilized free radical when acted on by HRP. Thus, they could not form free radicals and could not dimerize to precipitate out of solution.

# 7 Recommendations

Further research is required before any of the technologies and ideas developed in this investigation can be used in industrial applications. This further research must include optimizing the colour removal by ion exchange technology and further study into the precise structure of the specific components of pulp mill effluent colour.

## *7.1 On HRP*

The HRP enzyme was expected to remove colour from all the effluents tested, but was found to be ineffective. It may be of value to investigate the potential of other similar enzymes, such as *Arthromyces Ramoses* Peroxidase or Lignin Peroxidase for colour removal. However, these enzyme have the same basic mechanisms and could quite possibly be ineffective, as HRP was. A simple investigation should be conducted to determine if these other enzymes are effective. If so, an investigation similar to this one should then be undertaken to determine the best means of immobilization for the enzyme, in hopes of ultimately designing a reactor for removing colour. Because enzyme technology has been found to be effective in many other cases, further research is required.

## *7.2 On Colour*

The inability of HRP to remove any colour from the effluent led to the conclusion that the colour forming compounds were not phenolic or other aromatic

radical stabilizing structures. In order to better understand pulp mill colour and ensure the mills can maintain environmental compliance with colour regulations, it is important that future investigations include an effort to characterize the specific compounds which cause colour. This would likely be its own major investigation. However, it is very important to better understand these compounds.

### *7.3 On Ion Exchange Resins*

It was an unexpected result that the resin was a good remover of colour. Results of up to 85% removal of colour show a great amount of potential. Ion exchange technology is not widely used to remove contaminants from pulp mill waste. Perhaps this is due to high operating costs. Future research efforts should focus on removing the colour from the resin in order to reuse the resin. If it were found to be possible to achieve excellent colour removal from effluent and regenerate the resin back to its full removing potential, it may be found to be a cost effective technology which offers excellent environmental performance.

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# Appendices

# Appendix A

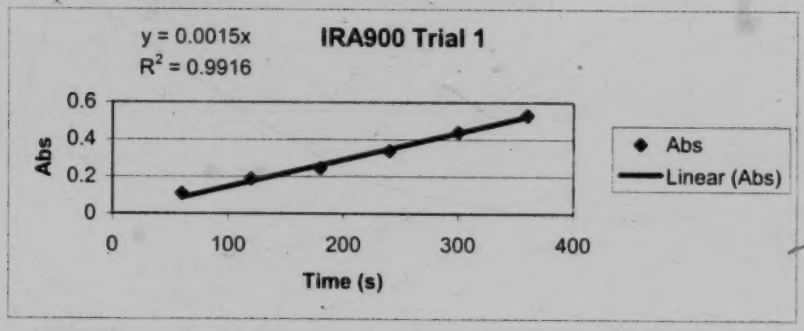
***Immobilized Enzyme Assay Results***

Listed below are the results of the immobilized enzyme assay trials. All times are in seconds and Abs = Absorbance.

**IRA 900**

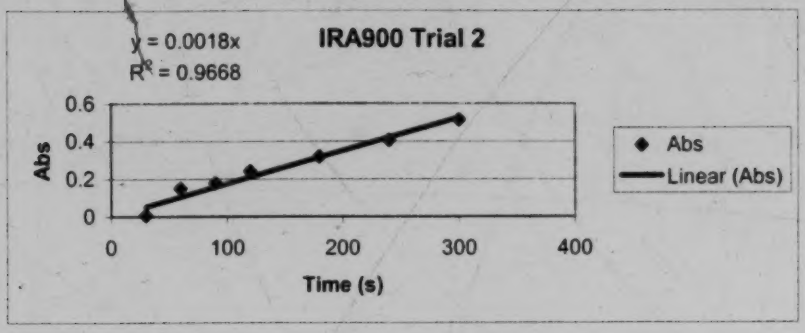
Trial 1

| Time | Abs |
|---|---|
| 60 | 0.10895 |
| 120 | 0.18807 |
| 180 | 0.2466 |
| 240 | 0.33866 |
| 300 | 0.43972 |
| 360 | 0.53065 |



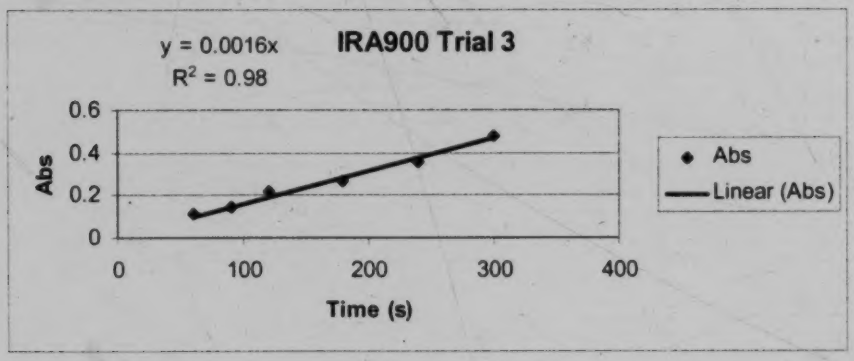

Trial 2

| Time | Abs |
|---|---|
| 30 | 0.007 |
| 60 | 0.14966 |
| 90 | 0.17735 |
| 120 | 0.24159 |
| 180 | 0.31882 |
| 240 | 0.40586 |
| 300 | 0.51534 |



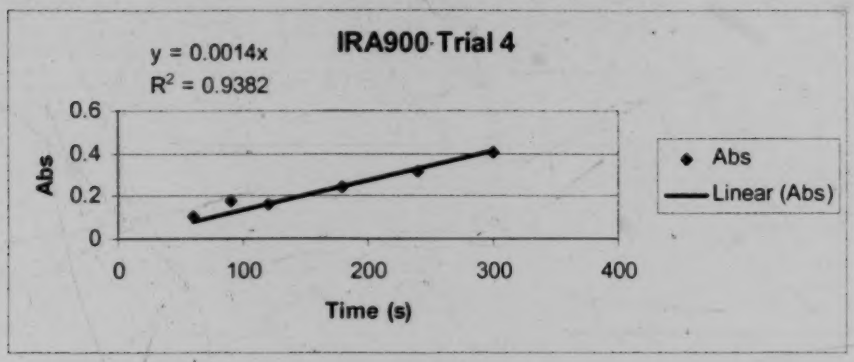

Trial 3

| Time | Abs |
|---|---|
| 60 | 0.11167 |
| 90 | 0.14451 |
| 120 | 0.21894 |
| 180 | 0.2678 |
| 240 | 0.35742 |
| 300 | 0.4758 |



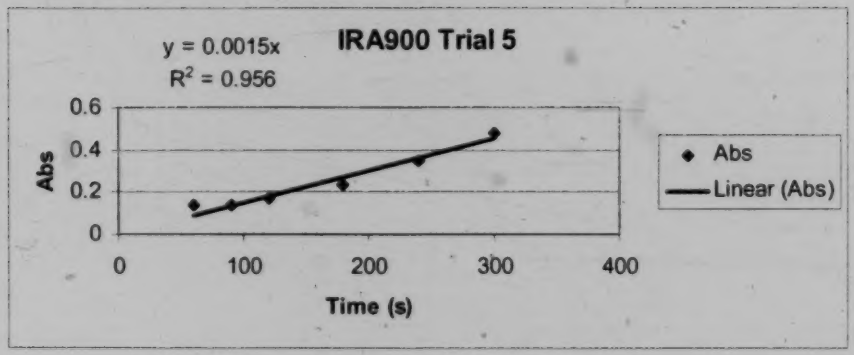

Trial 4

| Time | Abs |
|---|---|
| 60 | 0.10218 |
| 90 | 0.18131 |
| 120 | 0.16163 |
| 180 | 0.24719 |
| 240 | 0.31637 |
| 300 | 0.40927 |




Trial 5

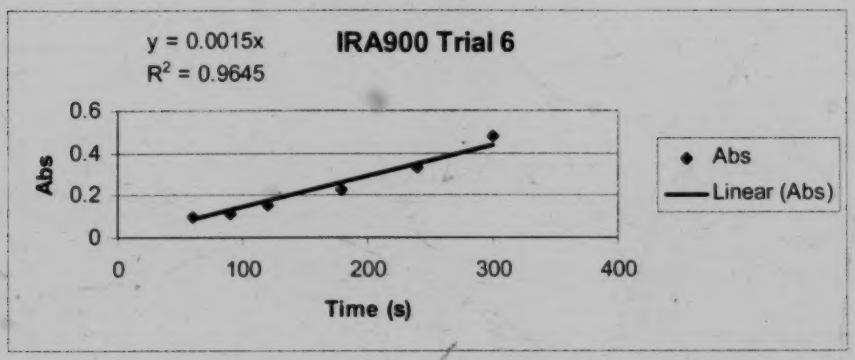
| Time | Abs |
|---|---|
| 60 | 0.1353 |
| 90 | 0.13542 |
| 120 | 0.16639 |
| 180 | 0.23774 |
| 240 | 0.34735 |
| 300 | 0.47676 |




Trial 6

| Time | Abs |
|---|---|
| 60 | 0.10092 |
| 90 | 0.1095 |
| 120 | 0.1574 |
| 180 | 0.23065 |

| | |
|---|---|
| 240 | 0.33278 |
| 300 | 0.47704 |



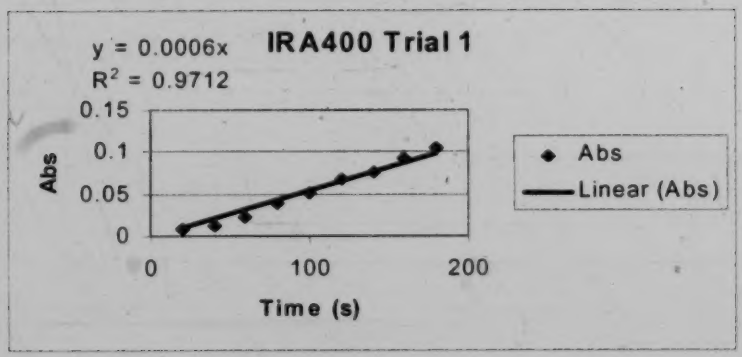

Activity Calculation

| Trial | Slope | Amount of Solid | Activity in cuvette | Total solid activity per gram |
|---|---|---|---|---|
| 1 | 0.0014 | 1 | 0.011830986 | 1.183098592 |
| 2 | 0.0017 | 1 | 0.014366197 | 1.436619718 |
| 3 | 0.0015 | 1 | 0.012676056 | 1.267605634 |
| 4 | 0.0012 | 1 | 0.010140845 | 1.014084507 |
| 5 | 0.0015 | 1 | 0.012676056 | 1.267605634 |
| 6 | 0.0016 | 1 | 0.013521127 | 1.352112676 |
| | | | Mean | 1.253521127 |
| | | | Standard deviation | 0.14555505 |
| | | | Confidence interval | 0.040079971 |

**IRA 400**

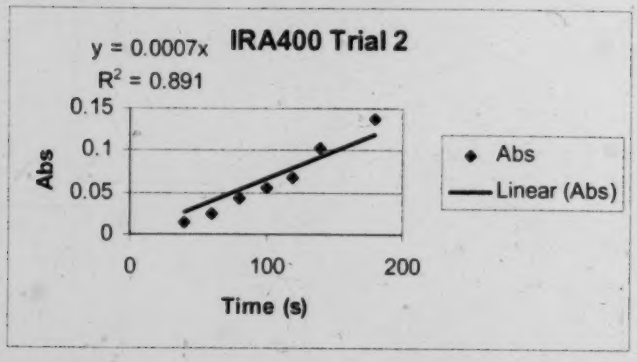
Trial 1

| Time | Abs |
|---|---|
| 20 | 0.008 |
| 40 | 0.013 |
| 60 | 0.023 |
| 80 | 0.04 |
| 100 | 0.051 |
| 120 | 0.068 |
| 140 | 0.076 |
| 160 | 0.092 |
| 180 | 0.105 |



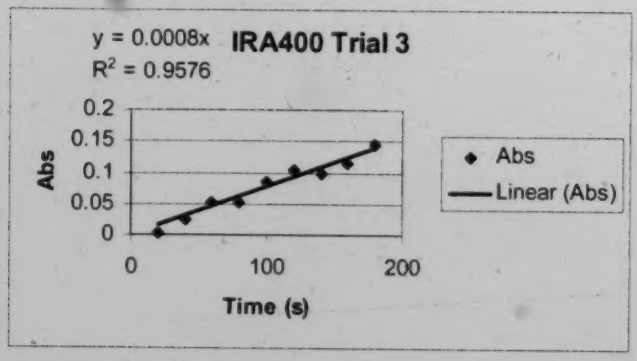

Trial 2

| Time | Abs |
|---|---|
| 40 | 0.0138 |
| 60 | 0.024 |
| 80 | 0.0436 |
| 100 | 0.0545 |
| 120 | 0.0668 |
| 140 | 0.10301 |
| 180 | 0.13865 |
| 20 | 0.0292 |




Trial 3

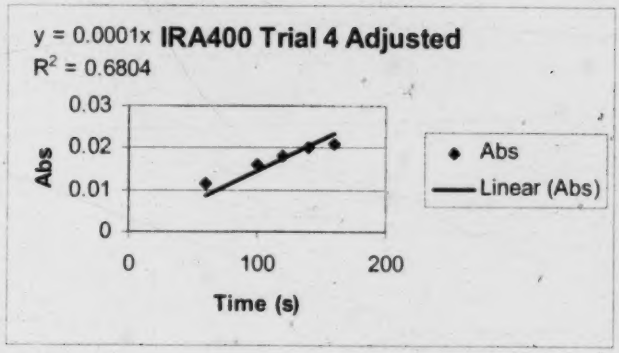
| Time | Abs |
|---|---|
| 20 | 0.00402 |
| 40 | 0.0239 |
| 60 | 0.0529 |
| 80 | 0.0526 |
| 100 | 0.0851 |
| 120 | 0.10537 |
| 140 | 0.0984 |
| 160 | 0.11606 |
| 180 | 0.144 |




Trial 4

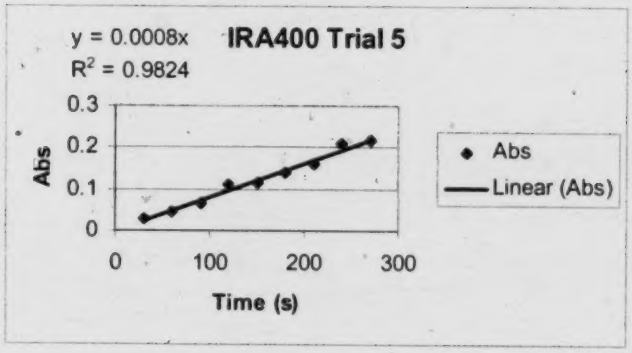
| Time | Abs |
|---|---|
| 20 | 0 |
| 40 | 0 |
| 60 | 0.0117 |
| 80 | 0 |
| 100 | 0.01605 |
| 120 | 0.0182 |
| 140 | 0.02014 |

| | |
|---|---|
| 160 | 0.02087 |
| 180 | 0.0405 |




Trial 5

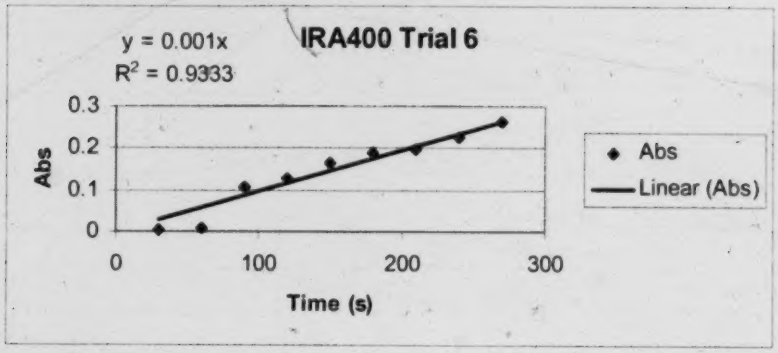
| Time | Abs |
|---|---|
| 30 | 0.0276 |
| 60 | 0.044 |
| 90 | 0.065 |
| 120 | 0.11033 |
| 150 | 0.11621 |
| 180 | 0.139 |
| 210 | 0.16094 |
| 240 | 0.20936 |
| 270 | 0.21738 |




Trial 6

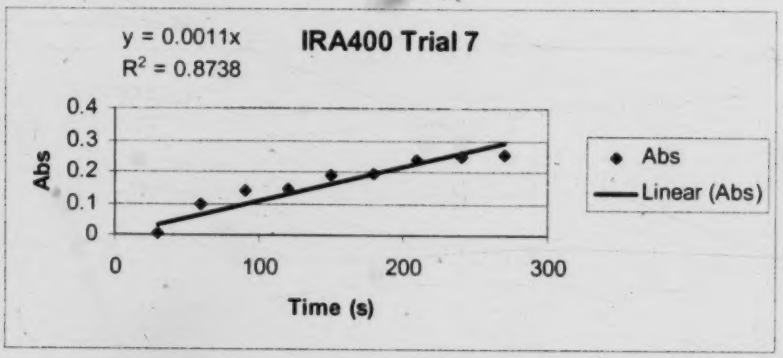
| Time | Abs |
|---|---|
| 30 | 0.004 |
| 60 | 0.009 |
| 90 | 0.10879 |
| 120 | 0.12566 |
| 150 | 0.16543 |

| | |
|---|---|
| 180 | 0.191 |
| 210 | 0.19614 |
| 240 | 0.22464 |
| 270 | 0.26335 |




Trial 7

| Time | Abs |
|---|---|
| 30 | 0.0075 |
| 60 | 0.0998 |
| 90 | 0.14161 |
| 120 | 0.14478 |
| 150 | 0.18901 |
| 180 | 0.19436 |
| 210 | 0.23986 |
| 240 | 0.24945 |
| 270 | 0.2529 |



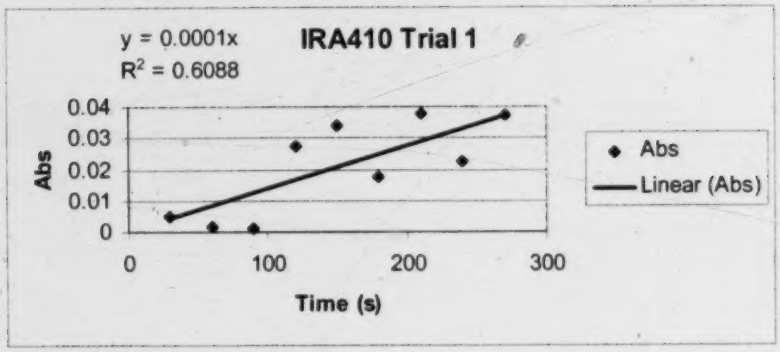

| Trial | Slope | Amount of Solid | Activity in cuvette | Total solid activity |
|---|---|---|---|---|
| 1 | 0.0006 | 0.5 | 0.005070423 | 1.014084507 |
| 2 | 0.0009 | 0.5 | 0.007605634 | 1.521126761 |
| 3 | 0.0008 | 0.5 | 0.006760563 | 1.352112676 |

| | | | | |
|---|---|---|---|---|
| 4 | 0.0002 | 0.5 | 0.001690141 | 0.338028169 |
| 5 | 0.0008 | 1 | 0.006760563 | 0.676056338 |
| 6 | 0.0011 | 1 | 0.009295775 | 0.929577465 |
| 7 | 0.0009 | 1 | 0.007605634 | 0.76056338 |
| 1a | 0.0008 | 0.5 | 0.006760563 | 1.352112676 |
| | | | Mean | 0.992957746 |
| | | | Stdev | 0.400852098 |
| | | | Conf int | 0.095590541 |

**IRA 410**

Trial 1

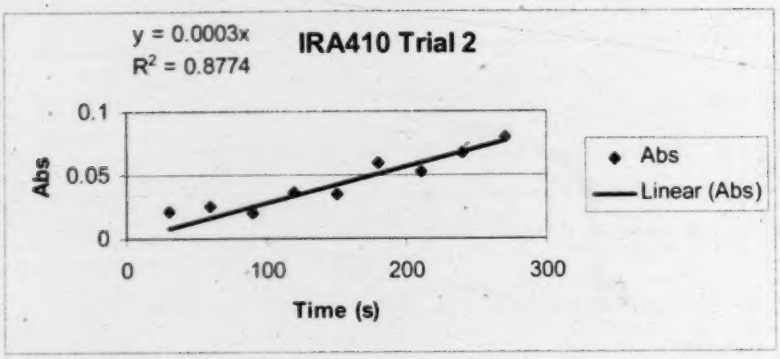
| Time | Abs |
|---|---|
| 30 | 0.00506 |
| 60 | 0.00187 |
| 90 | 0.00116 |
| 120 | 0.0272 |
| 150 | 0.0337 |
| 180 | 0.0177 |
| 210 | 0.0376 |
| 240 | 0.0226 |
| 270 | 0.037 |




Trial 2

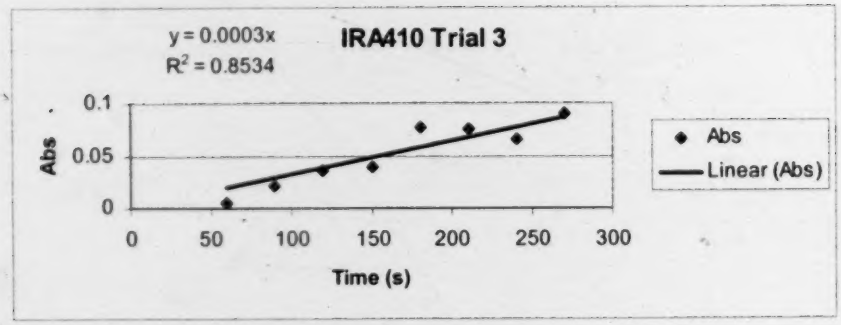
| Time | Abs |
|---|---|
| 30 | 0.02175 |
| 60 | 0.0256 |
| 90 | 0.0207 |
| 120 | 0.0362 |

| | |
|---|---|
| 150 | 0.0353 |
| 180 | 0.0598 |
| 210 | 0.0533 |
| 240 | 0.0676 |
| 270 | 0.0804 |




Trial 3

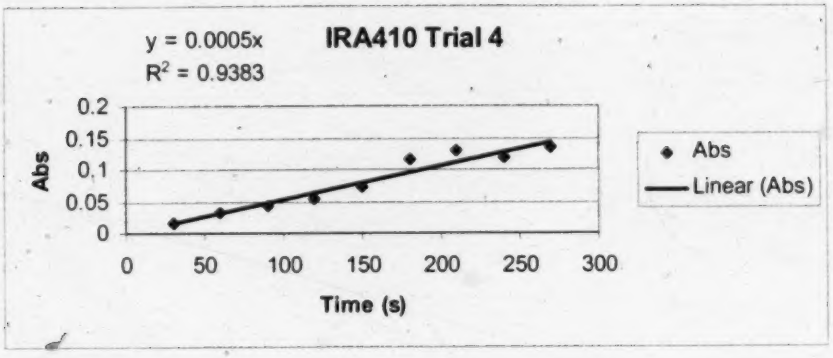
| Time | Abs |
|---|---|
| 60 | 0.00516 |
| 90 | 0.0207 |
| 120 | 0.0362 |
| 150 | 0.0397 |
| 180 | 0.0769 |
| 210 | 0.076 |
| 240 | 0.0651 |
| 270 | 0.0898 |




Trial 4

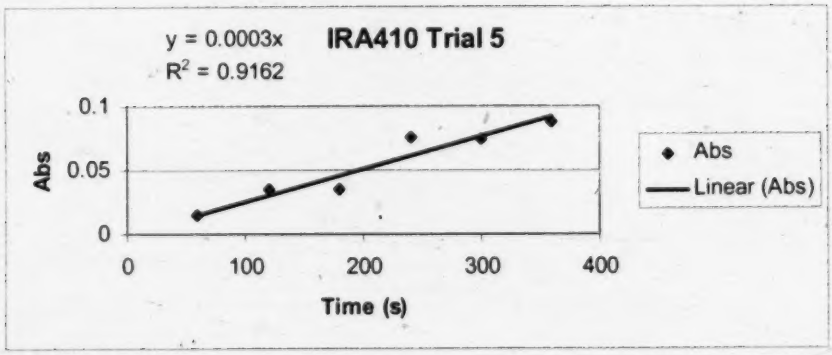
| Time | Abs |
|---|---|
| 30 | 0.0164 |
| 60 | 0.0314 |
| 90 | 0.043 |
| 120 | 0.0554 |

| | |
|---|---|
| 150 | 0.0735 |
| 180 | 0.11613 |
| 210 | 0.12897 |
| 240 | 0.11871 |
| 270 | 0.13402 |



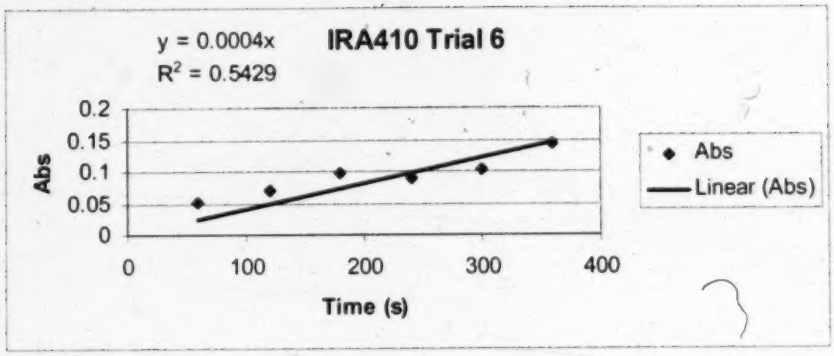

Trial 5

| Time | Abs |
|---|---|
| 60 | 0.0149 |
| 120 | 0.03456 |
| 180 | 0.0358 |
| 240 | 0.076 |
| 300 | 0.0739 |
| 360 | 0.088 |



Trial 6

| Time | Abs |
|---|---|
| 60 | 0.0522 |
| 120 | 0.0701 |
| 180 | 0.0979 |
| 240 | 0.0894 |
| 300 | 0.10375 |
| 360 | 0.14368 |




Calculation of Activity

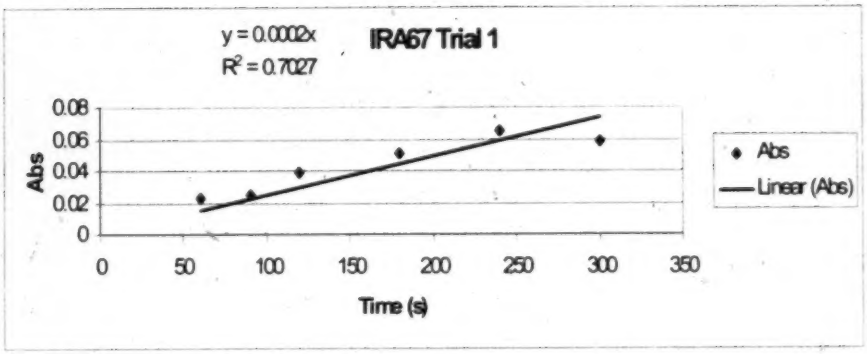
| Trial | Slope | Amount of Solid | Activity in cuvette | Total solid activity per gram |
|---|---|---|---|---|
| 1 | 0.0001 | 1 | 0.00084507 | 0.084507042 |
| 2 | 0.0002 | 1 | 0.001690141 | 0.169014085 |
| 3 | 0.0004 | 1 | 0.003380282 | 0.338028169 |
| 4 | 0.0005 | 1 | 0.004225352 | 0.422535211 |
| 5 | 0.0002 | 1 | 0.001690141 | 0.169014085 |
| 6 | 0.0003 | 1 | 0.002535211 | 0.253521127 |
| | | | Mean | 0.23943662 |
| | | | Stdev | 0.124390998 |
| | | | Conf int | 0.034252248 |

**IRA 67**

Trial 1

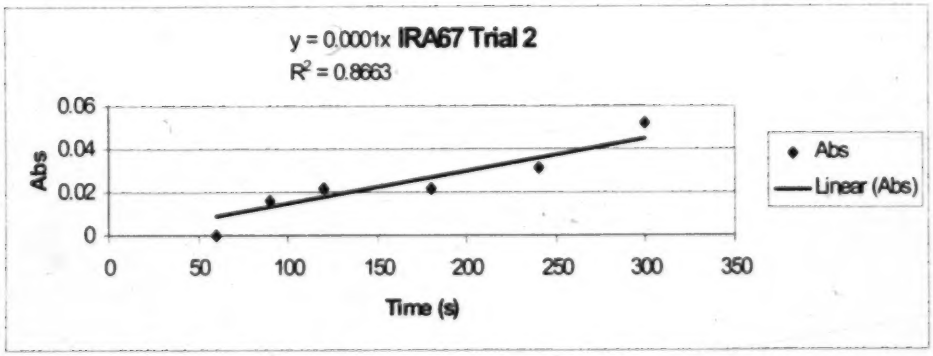
| Time | Abs |
|---|---|
| 60 | 0.0225 |
| 90 | 0.0246 |

| | |
|---|---|
| 120 | 0.0384 |
| 180 | 0.0513 |
| 240 | 0.0651 |
| 300 | 0.0584 |



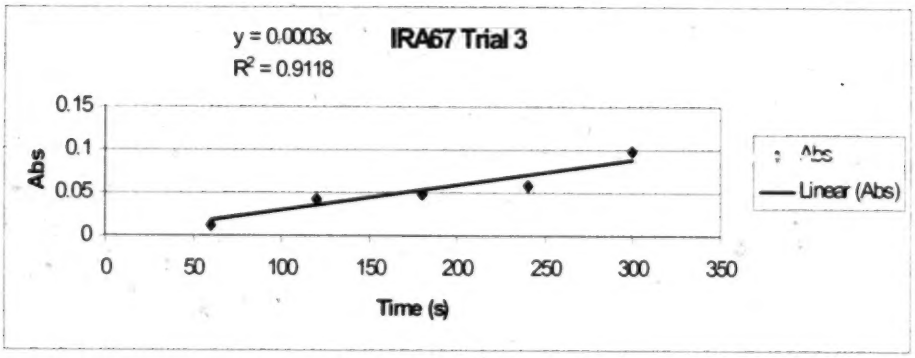

Trial 2

| Time | Abs |
|---|---|
| 60 | 0 |
| 90 | 0.0158 |
| 120 | 0.0216 |
| 180 | 0.0219 |
| 240 | 0.0309 |
| 300 | 0.0517 |



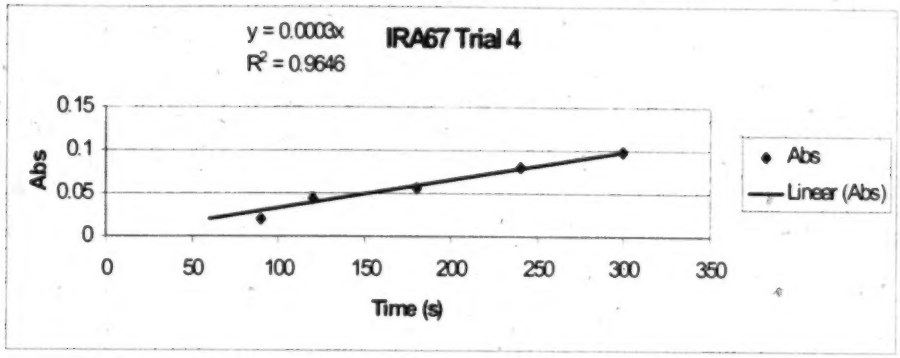

Trial 3

| Time | Abs |
|---|---|
| 60 | 0.0115 |
| 90 | |
| 120 | 0.0417 |
| 180 | 0.0482 |
| 240 | 0.0582 |
| 300 | 0.0977 |



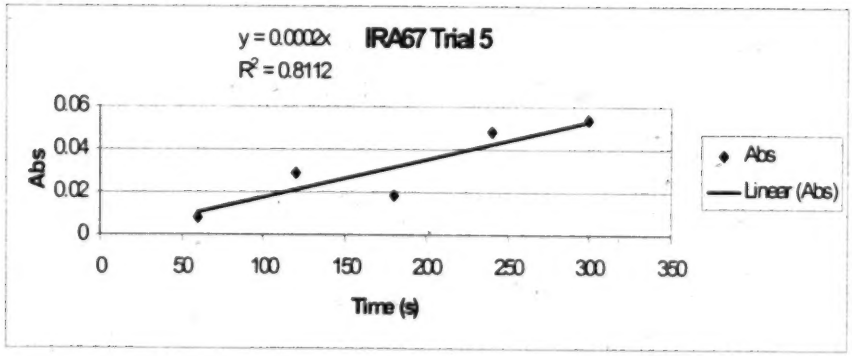

Trial 4

| Time | Abs |
|---|---|
| 60 | |
| 90 | 0.0197 |
| 120 | 0.0445 |
| 180 | 0.0562 |
| 240 | 0.0807 |
| 300 | 0.0987 |




Trial 5

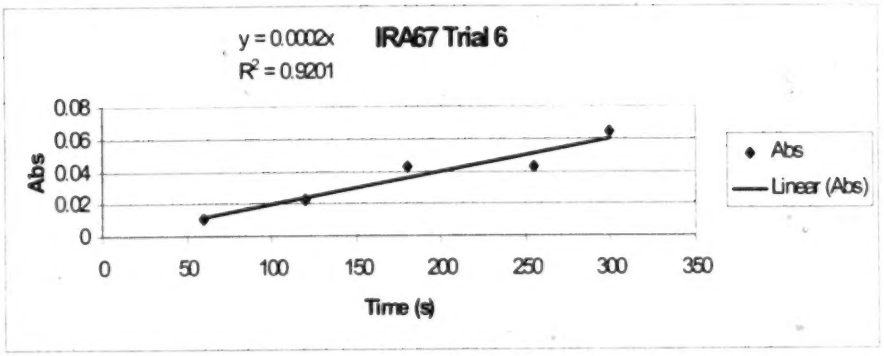
| Time | Abs |
|---|---|
| 60 | 0.00809 |
| 120 | 0.0291 |
| 180 | 0.0183 |
| 240 | 0.0481 |
| 300 | 0.0539 |




Trial 6

| Time | Abs |
|---|---|
| 60 | 0.0104 |
| 120 | 0.0219 |
| 180 | 0.0428 |

| | |
|---|---|
| 255 | 0.0427 |
| 300 | 0.064 |



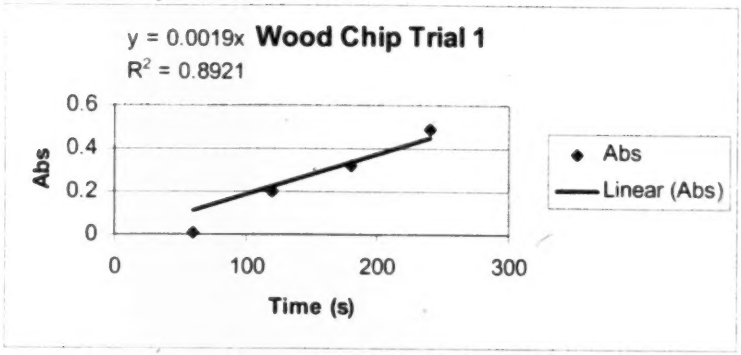

Calculation of Activity

| Trial | Slope | Amount of Solid | Activity in cuvette | Total solid activity per gram |
|---|---|---|---|---|
| 1 | 0.0002 | 2.004 | 0.001690141 | 0.084338366 |
| 2 | 0.0002 | 2.002 | 0.001690141 | 0.08442262 |
| 3 | 0.0003 | 2.998 | 0.002535211 | 0.084563418 |
| 4 | 0.0004 | 3.008 | 0.003380282 | 0.112376386 |
| 5 | 0.0002 | 2.9933 | 0.001690141 | 0.056464131 |
| 6 | 0.0002 | 3.0093 | 0.001690141 | 0.05616392 |
| | | | Mean | 0.079721473 |
| | | | Stdev | 0.021114291 |
| | | | Conf int | 0.005814022 |

**Wood Chips**

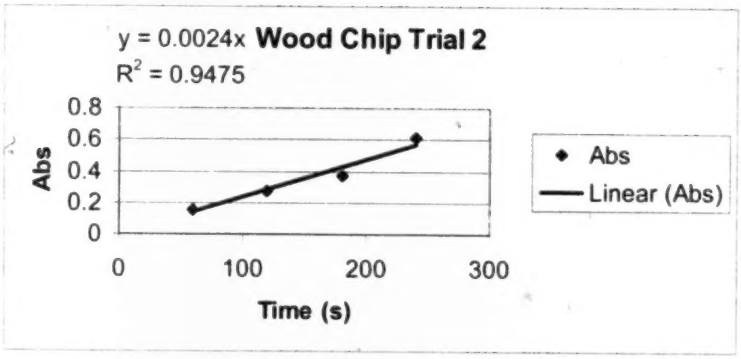
Trial 1

| Time | Abs |
|---|---|
| 60 | 0.00719 |
| 120 | 0.20267 |
| 180 | 0.32136 |
| 240 | 0.48873 |



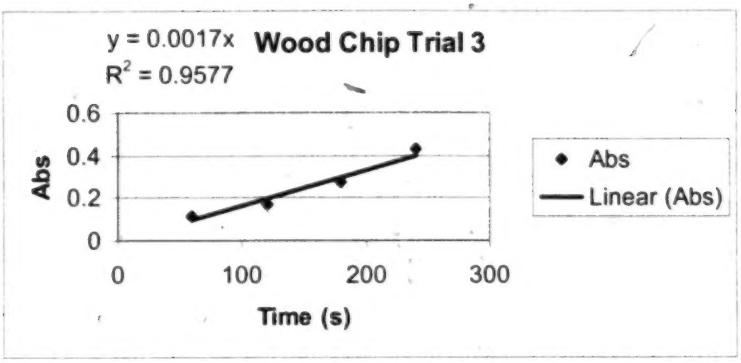

Trial 2

| Time | Abs |
|---|---|
| 60 | 0.15716 |
| 120 | 0.27852 |
| 180 | 0.36748 |
| 240 | 0.61558 |



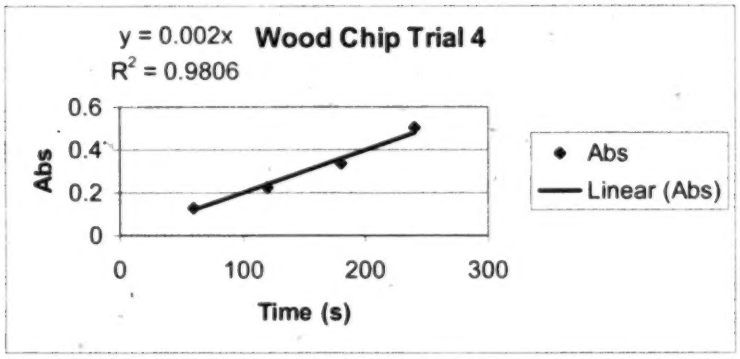

Trial 3

| Time | Abs |
|---|---|
| 60 | 0.11732 |
| 120 | 0.17067 |
| 180 | 0.27882 |
| 240 | 0.42788 |




Trial 4

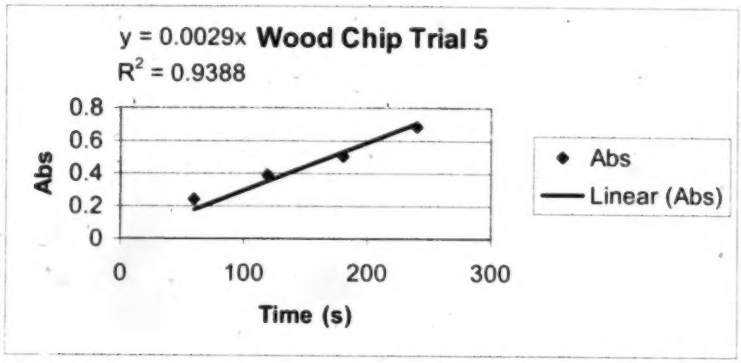
| Time | Abs |
|---|---|
| 60 | 0.13189 |
| 120 | 0.22544 |
| 180 | 0.33524 |
| 240 | 0.50266 |




Trial 5

| Time | Abs |
|---|---|
| 60 | 0.24325 |
| 120 | 0.38457 |

| | |
|---|---|
| 180 | 0.50298 |
| 240 | 0.6872 |




Calculation of Activity

| Trial | Slope | Amount of Solid | Activity in cuvette | Total solid activity per gram |
|---|---|---|---|---|
| 1 | 0.0026 | 4.1 | 0.021971831 | 0.535898317 |
| 2 | 0.0024 | 4.5 | 0.02028169 | 0.450704225 |
| 3 | 0.0017 | 5.4 | 0.014366197 | 0.266040689 |
| 4 | 0.002 | 4.7 | 0.016901408 | 0.359604435 |
| 5 | 0.0024 | 4.69 | 0.02028169 | 0.432445419 |
| | | | Mean | 0.408938617 |
| | | | Stdev | 0.101574333 |
| | | | Conf int | 0.027969462 |

***Shear Test Results***

Shear test results report the soluble HRP activity in the supernatant solution after the shear had been applied. In the data below, slope is derived from the spectrophotometer's calculation of the change in absorbance over time. Amount of solid is in grams.

**IRA 900**

Desorption

| Trial | Slope | Amount of Solid | Activity in cuvette | Activity in Supernatant per gram | Removal % |
|---|---|---|---|---|---|
| 1 | 0.097 | 2.002 | 0.01366197 | 0.170604044 | 117.209 |
| 2 | 0.096 | 2.002 | 0.01352112 | 0.168845239 | 116.000 |
| 3 | 0.095 | 2.002 | 0.01338028 | 0.167086435 | 114.792 |
| 4 | 0.098 | 2.008 | 0.01380281 | 0.17184782 | 118.063 |
| 5 | 0.099 | 2.008 | 0.01404225 | 0.174828854 | 120.111 |
| 6 | 0.095 | 2.008 | 0.01338028 | 0.166587172 | 114.449 |
| | | | | Average: | 116.771 |

**IRA 400**

Desorption

| Trial | Slope | Amount of Solid | Activity in cuvette | Activity in Supernatant per gram | Removal % |
|---|---|---|---|---|---|
| 1 | 0.00792 | 2.0072 | 0.00111549 | 0.013893645 | 1.3992 |
| 2 | 0.00865 | 2.0072 | 0.00121831 | 0.015174246 | 1.5282 |
| 3 | 0.0106 | 2.0072 | 0.00149295 | 0.01859503 | 1.87269 |
| 4 | 0.00918 | 2.0089 | 0.00129296 | 0.01609037 | 1.62045 |
| 5 | 0.00926 | 2.0089 | 0.00130422 | 0.016230591 | 1.63457 |
| 6 | 0.00982 | 2.0089 | 0.00138309 | 0.017212138 | 1.73342 |
| | | | | Average | 1.63142 |
| | | | | Standard deviation | 0.16329 |

**IRA 410**

Desorption

| Trial | Slope | Amount of Solid | Activity in cuvette | Activity in Supernatant per gram | Removal % |
|---|---|---|---|---|---|
| 1 | 0.0157 | 2.0006 | 0.002211268 | 0.027632555 | 11.5406 |
| 2 | 0.0166 | 2.0006 | 0.002338028 | 0.029216587 | 12.2022 |
| 3 | 0.0147 | 2.0006 | 0.002070423 | 0.02587252 | 10.8055 |
| 4 | 0.014 | 2.0008 | 0.001971831 | 0.024638032 | 10.29 |
| 5 | 0.0132 | 2.0008 | 0.001859155 | 0.023230145 | 9.70200 |
| 6 | 0.0127 | 2.0008 | 0.001788732 | 0.022350215 | 9.33450 |
| | | | | average | 10.6458 |
| | | | | Std. Dev. | 1.09412 |

**IRA 67**

Desorption

| Trial | Slope | Amount of Solid | Activity in cuvette | Activity in Supernatant per gram | Removal % |
|---|---|---|---|---|---|
| 1 | 0.002 | 2.0003 | 0.000282 | 0.003520599 | 16.674 |
| 2 | n/a | n/a | n/a | n/a | n/a |
| 3 | 0.005 | 2.0003 | 0.000704 | 0.008801497 | 41.6850 |
| 4 | 0.01 | 2.0027 | 0.001408 | 0.017581898 | 83.2701 |
| 5 | 0.005 | 2.0027 | 0.000704 | 0.008790949 | 41.6351 |
| 6 | 0.005 | 2.0027 | 0.000704 | 0.008790949 | 41.6351 |

# Appendix B

***Colour Removal Trial Results***

**Factorial Experiment Results**

| Trial | 1 PEG Level | 2 H2O2 Level | 3 Enzyme Dose | Average Colour Result (pccu) | Colour Removed (pccu) | % Colour Removed |
|---|---|---|---|---|---|---|
| 1 | - | - | - | 490 | 575 | 53.99% |
| 2 | 0 | - | - | 460 | 605 | 56.81% |
| 3 | + | - | - | 465 | 600 | 56.34% |
| 4 | - | 0 | - | 330 | 735 | 69.01% |
| 5 | 0 | 0 | - | 415 | 650 | 61.03% |
| 6 | + | 0 | - | 365 | 700 | 65.73% |
| 7 | - | + | - | 300 | 765 | 71.83% |
| 8 | 0 | + | - | 310 | 755 | 70.89% |
| 9 | + | + | - | 315 | 750 | 70.42% |
| 10 | - | - | 0 | 290 | 775 | 72.77% |
| 11 | 0 | - | 0 | 320 | 745 | 69.95% |
| 12 | + | - | 0 | 330 | 735 | 69.01% |
| 13 | - | 0 | 0 | 250 | 815 | 76.53% |
| 14 | 0 | 0 | 0 | 255 | 810 | 76.06% |
| 15 | + | 0 | 0 | 280 | 785 | 73.71% |
| 16 | - | + | 0 | 195 | 870 | 81.69% |
| 17 | 0 | + | 0 | 220 | 845 | 79.34% |
| 18 | + | + | 0 | 220 | 845 | 79.34% |
| 19 | - | - | + | 180 | 885 | 83.10% |
| 20 | 0 | - | + | 180 | 885 | 83.10% |
| 21 | + | - | + | 180 | 885 | 83.10% |
| 22 | - | 0 | + | 180 | 885 | 83.10% |
| 23 | 0 | 0 | + | 155 | 910 | 85.45% |
| 24 | + | 0 | + | 150 | 915 | 85.92% |
| 25 | - | + | + | 130 | 935 | 87.79% |
| 26 | 0 | + | + | 145 | 920 | 86.38% |
| 27 | + | + | + | 145 | 920 | 86.38% |

**Sample Calculation of Colour**

Trial 1: all low levels
No dilution for test.

Abs at 465nm: 0.13054, 0.13073, 0.13095
Avg abs: = (0.13054+0.13073+0.13095) /3 = 0.13074.
Abs of Pt Co Std: 0.1336

Colour = (Abs Sample / Abs Standard) * 500 pccu
=(0.13074 / 0.1336) * 500 pccu
=490 pccu
The colour results listed in the factorial result chart are averaged between two

replicate sets of results. The list of replicates is below.

| Colour Result A | Colour Result B |
|---|---|
| 490 | 490 |
| 460 | 485 |
| 465 | 470 |
| 330 | 315 |
| 415 | 425 |
| 365 | 410 |
| 75 | 300 |
| 310 | 300 |
| 315 | 310 |
| 290 | 310 |
| 320 | 300 |
| 330 | 345 |
| 160 | 250 |
| 255 | 270 |
| 280 | 260 |
| 195 | 240 |
| 220 | 240 |
| 220 | 230 |
| 180 | 190 |
| 180 | 185 |
| 180 | 180 |
| 180 | 170 |
| 155 | 165 |
| 150 | 165 |
| 130 | 140 |
| 145 | 135 |
| 145 | 145 |

**Yates' Algorithm: Calculating Effects and Interactions**

The following chart displays the list of numbered columns used in Yates' Algorithm to determine effects and interactions from a $3^3$ factorial design. Column 0 lists the trial number. Column 1 is result of colour removal trials. Columns labeled 2,3 and 4 are intermediate calculation steps (Box and Draper 1987). Column 5 lists effect names, where 0 represents no factor , 1 represents PEG level, 2 represents $H_2O_2$ level and 3 represents enzyme/resin dose. Higher order interactions are written as raised powers (1^2 = $1^2$). Column 6 is a divisor used in calculating orthogonal effects listed in Column 7. Column 8 lists the mean square of the effect calculated.

Column 1

| Trial "0" | 8.1.1.1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| 1 | 575 | 1780 | 6135 | 21500 | 0 0 0 | 27 | 796.2963 | 17120370 |
| 2 | 605 | 2085 | 7225 | -105 | 1 0 0 | 18 | -5.833 | 612.5 |
| 3 | 600 | 2270 | 8149 | 125 | 1^2 0 0 | 54 | 2.314 | 289.35 |
| 4 | 735 | 2255 | -25 | 915 | 0 2 0 | 18 | 50.83333 | 46512.5 |
| 5 | 650 | 2410 | -95 | -40 | 1 2 0 | 12 | -3.33333 | 133.333 |
| 6 | 700 | 2560 | 15 | 60 | 1^2 2 0 | 36 | 1.666667 | 100 |
| 7 | 765 | 2655 | 105 | -115 | 0 2^2 0 | 54 | -2.12963 | 244.907 |
| 8 | 755 | 2710 | 25 | 0 | 1 2^2 0 | 36 | 0 | 0 |
| 9 | 750 | 2775 | -5 | -160 | 1^2 2^2 0 | 108 | -1.48148 | 237.037 |
| 10 | 775 | 25 | 490 | 2005 | 0 0 3 | 18 | 111.3888 | 223334.7 |
| 11 | 745 | -35 | 305 | 40 | 1 0 3 | 12 | 3.333333 | 133.3333 |
| 12 | 735 | -15 | 120 | -110 | 1^2 0 3 | 36 | -3.05556 | 336.1111 |
| 13 | 815 | -40 | -40 | -370 | 0 2 3 | 12 | -30.8333 | 11408.33 |
| 14 | 810 | -30 | 15 | 25 | 1 2 3 | 8 | 3.125 | 78.125 |
| 15 | 785 | -25 | -15 | -25 | 1^2 2 3 | 24 | -1.04167 | 26.04167 |
| 16 | 870 | 0 | 40 | 130 | 0 2^2 3 | 36 | 3.611111 | 469.4444 |
| 17 | 845 | 30 | 5 | -155 | 1 2^2 3 | 24 | -6.45833 | 1001.042 |
| 18 | 845 | -15 | 15 | 355 | 1^2 2^2 3 | 72 | 4.930555 | 1750.347 |
| 19 | 885 | -35 | -120 | -175 | 0 0 3^2 | 54 | -3.24074 | 567.1296 |
| 20 | 885 | 135 | -5 | 180 | 1 0 3^2 | 36 | 5 | 900 |
| 21 | 885 | 5 | 10 | 50 | 1^2 0 3^2 | 108 | 0.462963 | 23.14815 |
| 22 | 885 | 20 | 80 | 0 | 0 2 3^2 | 36 | 0 | 0 |
| 23 | 910 | -20 | -5 | -85 | 1 2 3^2 | 24 | -3.54167 | 301.0417 |
| 24 | 915 | 25 | -75 | 45 | 1^2 2 3^2 | 72 | 0.625 | 28.125 |
| 25 | 935 | 0 | -300 | -100 | 0 2^2 3^2 | 108 | -0.92596 | 92.59259 |
| 26 | 920 | -20 | 85 | 15 | 1 2^2 3^2 | 72 | 0.208333 | 3.125 |
| 27 | 920 | 15 | 55 | -415 | 1^2 2^2 3^2 | 216 | -1.92126 | 797.338 |

Sum: 17409750

**ANOVA Results**

ANOVA is used to determine which effects and interactions are significant. Listed below are the replicate colour results to determine the mean square of errors.

| Colour Result | Repeat | Difference |
|---|---|---|
| 490 | 490 | 0 |
| 460 | 485 | 25 |
| 465 | 470 | 5 |
| 330 | 315 | 15 |
| 415 | 425 | 10 |
| 365 | 410 | 45 |
| 275 | 300 | 25 |
| 75 | 300 | 225 |
| 315 | 310 | 5 |
| 290 | 310 | 20 |
| 320 | 300 | 20 |
| 330 | 345 | 15 |
| 160 | 250 | 90 |
| 255 | 270 | 15 |
| 280 | 260 | 20 |
| 195 | 240 | 45 |
| 220 | 240 | 20 |
| 220 | 230 | 10 |
| 180 | 190 | 10 |
| 180 | 185 | 5 |
| 180 | 180 | 0 |
| 180 | 170 | 10 |
| 155 | 165 | 10 |
| 150 | 165 | 15 |
| 130 | 140 | 10 |
| 145 | 135 | 10 |
| 145 | 145 | 0 |
| Mean Square | error | 16825 |

<continued next page>

Next, the effects and interactions are listed along with the mean squares of effects and errors. If the value for mean square of effect / mean square of error is greater than the critical F value for $\alpha = 0.5$ for sample size of 30 (1.89), the effect or interaction is significant.

| | Orth. Effect | MS effect | MS error | MS eff/MS err | Significant? |
|---|---|---|---|---|---|
| 0 0 0 | 796.296 | 17120370.370 | 16825 | 1017.555 | |
| 1 0 0 | -5.833 | 612.500 | 16825 | 0.036 | No |
| 1^2 0 0 | 2.315 | 289.352 | 16825 | 0.017 | No |
| 0 2 0 | 50.833 | 46512.500 | 16825 | 2.764 | Yes |
| 1 2 0 | -3.333 | 133.333 | 16825 | 0.008 | No |
| 1^2 2 0 | 1.667 | 100.000 | 16825 | 0.006 | No |
| 0 2^2 0 | -2.130 | 244.907 | 16825 | 0.015 | No |
| 1 2^2 0 | 0.000 | 0.000 | 16825 | 0.000 | No |
| 1^2 2^2 0 | -1.481 | 237.037 | 16825 | 0.014 | No |
| 0 0 3 | 111.389 | 223334.722 | 16825 | 13.274 | Yes |
| 1 0 3 | 3.333 | 133.333 | 16825 | 0.008 | No |
| 1^2 0 3 | -3.056 | 336.111 | 16825 | 0.020 | No |
| 0 2 3 | -30.833 | 11408.333 | 16825 | 0.678 | No |
| 1 2 3 | 3.125 | 78.125 | 16825 | 0.005 | No |
| 1^2 2 3 | -1.042 | 26.042 | 16825 | 0.002 | No |
| 0 2^2 3 | 3.611 | 469.444 | 16825 | 0.028 | No |
| 1 2^2 3 | -6.458 | 1001.042 | 16825 | 0.059 | No |
| 1^2 2^2 3 | 4.931 | 1750.347 | 16825 | 0.104 | No |
| 0 0 3^2 | -3.241 | 567.130 | 16825 | 0.034 | No |
| 1 0 3^2 | 5.000 | 900.000 | 16825 | 0.053 | No |
| 1^2 0 3^2 | 0.463 | 23.148 | 16825 | 0.001 | No |
| 0 2 3^2 | 0.000 | 0.000 | 16825 | 0.000 | No |
| 1 2 3^2 | -3.542 | 301.042 | 16825 | 0.018 | No |
| 1^2 2 3^2 | 0.625 | 28.125 | 16825 | 0.002 | No |
| 0 2^2 3^2 | -0.926 | 92.593 | 16825 | 0.006 | No |
| 1 2^2 3^2 | 0.208 | 3.125 | 16825 | 0.000 | No |
| 1^2 2^2 3^2 | -1.921 | 797.338 | 16825 | 0.047 | No |

According to the ANOVA analysis, only effect "2" and "3" ($H_2O_2$ dose and Enzyme dose) are significant.

**Method Blank Trial Results**

Below are the results to the Method Blank Trials.

| Amount of Resin: | 5g | 15g | 30g | Abs of Std |
|---|---|---|---|---|
| Absorbance: | 0.12543 | 0.074 | 0.067 | 0.1331 |
| | 0.12593 | 0.073 | 0.067 | 0.13337 |
| | 0.12606 | 0.073 | 0.068 | 0.13342 |
| | 0.12834 | 0.079 | 0.051 | 0.13329 7 |
| | 0.1274 | 0.079 | 0.051 | |
| | 0.128 | 0.079 | 0.051 | |
| Avg. Absorbance: | 0.12686 | 0.076167 | 0.059167 | |
| colour (pccu): | 475.85586 | 285.7036 | 221.936 | |
| **Colour removed (pccu)** | **589.14414** | **779.2964** | **843.064** | |

Blank: High H2O2, 0 PEG, 5g resin: 370

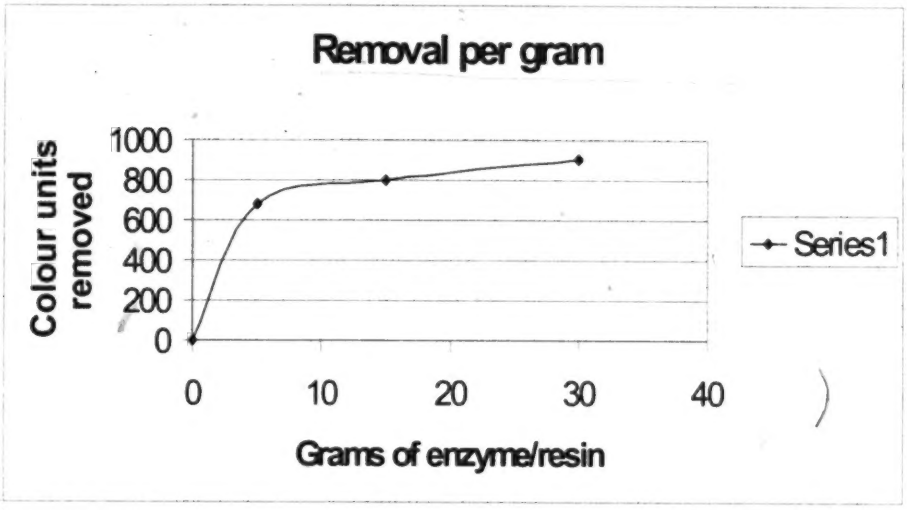
Blank: med H2O2, 0 PEG, 5g resin: 405

Blank: 100 ml eff, 30g resin 135

Rpt: 250
rpt: 190
15g resin 275
rpt: 295
5g resin 470
rpt: 480

**Plot to Test Linearity of Colour Removal**

As discussed in Section 5.6, the colour removal was plotted against increasing resin dose. The resulting graph is not linear, suggesting that perhaps removal of colour via resin is asymptotic.



***Colour Removal Trials on Other Wastes***

Below are the results of the colour removal trials on other pulp mill effluents. In the following data WW refers to Weldwood effluent, WH refers to Weyerhaeuser effluent, "eff" refers to final effluent and BP refers to bleach plant effluent, ie WW BP = Weldwood bleach plant effluent.

| Effluent: | WW eff | WW eff (2) | WH BP | WH BP (2) | WW BP | WW BP (2) | Std |
|---|---|---|---|---|---|---|---|
| Absorbance: | 0.0282 | 0.03 | 0.098 | 0.098 | 0.008 | 0.009 | 0.13124 |
| | 0.0286 | 0.029 | 0.098 | 0.098 | 0.008 | 0.009 | 0.13201 |
| | 0.0292 | 0.03 | 0.098 | 0.098 | 0.008 | 0.009 | 0.13198 |
| Average Absorbance: | 0.0286667 | 0.029667 | 0.098 | 0.098 | 0.008 | 0.009 | 0.131743 |
| | | 0.029167 | | 0.098 | | 0.0085 | |
| | Colour: | 110.695 | | 371.9353 | | 32.259697 | |
| | Orig Col: | 419.4612 | | 1363.91 | | 589.29825 | |
| | Units rem: | 308.7661 | | 991.9744 | | 557.03855 | |
| | % Removal: | 73.61018 | | 72.73021 | | 94.525744 | |

| WW eff | WH BP | WW BP | Std |
|---|---|---|---|
| 0.1103 | 0.36371 | 0.157 | 0.13299 |
| 0.1125 | 0.36204 | 0.15532 | 0.13305 |
| 0.11193 | 0.36265 | 0.15794 | 0.13296 |
| 0.111577 | 0.3628 | 0.156753 | 0.133 |